FETAL WEIGHT AND ORAL COLONIZATION WITH *PORPHYROMONAS GINGIVALIS*

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DEDICATION

This thesis is dedicated to my family, including the Fischers, Fairbanks, and Santos. It is also dedicated to my family members who walk on four legs instead of two. Also to the cows, who started it all.

I would also like to acknowledge my family not related by blood, including the Manga Anime Society and Anime Detour.

Lastly, it would be remiss of me not to include all the students I've had the chance to mentor through these years of my thesis work.

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ABSTRACT

Periodontitis in humans is associated with adverse pregnancy outcomes (APOs). However, periodontitis treatment trials (scaling and root planing) have not been successful at reducing the risk of APOs. Oral colonization with the periodontal pathogen *Porphyromonas gingivalis* leads to low fetal weight in C57BL/6J mice. We speculated that spread of *P. gingivalis* to the placenta of the C57BL/6J mouse would alter the placental microbiome, inducing local inflammation. We hypothesized that the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ in the uterine-draining (para-aortic) and oral draining (cervical) lymph nodes (PaLN-CLN) would correlate with low fetal weight in C57BL/6J mice. We discovered that frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 in PaLN-CLN of C57BL/6J (Jackson Labs) or C57BL/6UofM (In-House Bred) mice was not correlated with reduced fetal weight. Decreasing fetal weight was correlated with increasing amounts of *P. gingivalis* DNA in the placentas of C57BL/6J and C57BL/6UofM dams. BALB/cJ (Jackson), C57BL/6NCrl, and BALB/cAnNCrl (Charles River) mice harbored *P. gingivalis* DNA in their placentas but did not experience low fetal weight in response to oral colonization with *P. gingivalis*. Therefore, these results suggest that both microbiome and genetics are important in determining fetal weight outcomes in response to oral colonization with *P. gingivalis*.

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Key Abbreviations

APOs: adverse pregnancy outcomes

CAL: clinical attachment level

CLN: cervical lymph node

E17: embryonic day 17

IL-#: interleukin #

IFN-γ: interferon gamma

LBW: low birth weight

MOTOR: Maternal Oral Therapy to Reduce Obstetric Risk Study

NIDCR: National Institute of Dental and Craniofacial Research

OPT: Obstetrics and Periodontal Therapy Trial

MHC: major histocompatibility complex

PaLN: para-aortic (also called lumbar) lymph nodes

PD: probing depth

PLBW: preterm and low birth weight

PROM: premature rupture of membranes

PTB: preterm birth

PTL: preterm labor

RANK-L: receptor activator of nuclear factor kappaβ ligand

SFB: segmented filamentous bacterium

SI-LP: small intestine lamina propria

SRP: scaling and root planing

TLR: toll-like receptor

Preamble

Women with periodontitis during pregnancy are predicted to be at higher risk of adverse pregnancy outcomes (APOs) based on data obtained from observational studies. Investigators have attempted to reduce the risk of APOs with standard non-surgical periodontal treatment [Scaling and Root Planing (SRP)] delivered in the second trimester of pregnancy. One such treatment study was the Obstetrics and Periodontal Therapy Trial (OPT), funded by the National Institute of Dental and Craniofacial Research (NIDCR). OPT included the Hennepin County Medical Center in Minneapolis in collaboration with University of Minnesota periodontists and clinical researchers. Unfortunately, while the SRP treatment significantly improved periodontal health, it failed to reduce the risk of APOs. These findings were corroborated by a second NIDCR funded study, Maternal Oral Therapy to Reduce Obstetric Risk Study (MOTOR) and by meta-analysis of multiple large, well-controlled clinical trials testing identical treatment and a variety of similar APOs.

Some critics maintain that the failure of treatment trials to reduce the risk of APOs indicate that there is no association between periodontal disease and APOs. This criticism applies a faulty logic because it assumes *a priori* that microorganisms residing only in the periodontal pocket are the sole contributors to APOs. The thesis presented here provides evidence that SRP trials failed to reduce risk of APOs because they did not prevent or treat the spread of periodontal pathogens to the placental tissue. Once in the placental tissue,

periodontal pathogens could either mediate direct placental tissue damage or elicit an inflammatory immune response that impairs the fetoplacental unit, eventually leading to APOs. Additionally, this thesis produces data suggesting that development of APOs in response to oral pathogens is modulated by both maternal microbiome and genetics.

Relevant Background

Periodontal Disease and Periodontitis

The broad term “periodontal disease” includes both gingivitis and periodontitis. Gingivitis always precedes periodontitis but not all gingivitis cases progress to periodontitis. Periodontitis is a complex chronic disease, induced by oral microorganisms eliciting a chronic inflammatory response in the susceptible host. Periodontitis eventually results in inflammation-mediated destruction of alveolar bone around the root of the tooth and loss of connective tissue attachment to the root surface. Ultimately periodontitis leads to tooth loss (1, 2). To conclude that APOs are associated with severe periodontitis, it is critical to measure periodontitis accurately in clinical trials and observational studies.

Measuring Periodontitis

Periodontitis is defined as a permanent loss of clinical attachment level (CAL). CAL is commonly associated with deep periodontal pockets. The standard definitions of periodontal disease severity are based on the frequency of periodontal sites fitting within a predefined CAL category measured in millimeters: slight/mild periodontitis (CAL 1-2 mm); moderate periodontitis (CAL 3-4 mm); severe periodontitis (CAL ≥5 mm) (3-7). The extent of periodontitis is considered localized when the aforementioned, predefined amount of CAL is present in less than 30% of sites versus generalized if greater than 30% of sites

measured are affected (3-6). The most thorough examination measures six sites per tooth, in all teeth or a in selected subset of teeth in the mouth. Measuring fewer sites or fewer teeth in a clinical trial may save time but also may skew the estimate of periodontitis extent and severity.

Probing depth (PD) is another measure of periodontal disease. The difference between CAL and PD can be difficult to define to the non-dentist. The two measures have several similarities. Both are related to the loss of attachment of fibers that normally connect the cementum covering the dentin of the tooth root, to the adjacent gingiva and surrounding bone (7). CAL and PD are measured in millimeters by inserting a calibrated probe into the "pocket" between the tooth and gingiva until it reaches the bottom of the gingival sulcus, where the probe tip is stopped by the connective tissues attaching to the cementum of the root (attachment point) (1, 7, 8).

CAL is measured from the bottom of the pocket to the cemento-enamel junction, which is the anatomically defined fixed reference point on the tooth (9). In contrast PD is measured from the bottom of the pocket to the top of the gingiva, and thus is dependent on the state of inflammation or recession of the gingiva at the time the measurement is taken (9). Clinical studies have suggested that measuring PD without measuring CAL may lead clinicians to overlook sites with persistent, progressive loss of CAL over time without changes on the depth of the gingival pocket (9).

Accuracy of CAL or PD measurement depends on the skill of the examiner, the shape and size of the probe tip, the pressure with which the tip is applied and whether a standard or constant pressure probe is used, the angle that the probe is inserted, the presence of subgingival deposits on the root surface, and the extent of inflammation (10-12).

A third, less invasive method of measuring periodontitis severity is to use radiographs to evaluate the loss of bone support around the tooth. Radiographs' reproducibility varies depending on the incident angle, the operator and specific instrumentation used. When all the pros and cons are considered, CAL is generally the preferred method of clinicians to diagnose periodontitis.

Generally, due to the amount of technical measurement-error associated with the variables listed above, it is *not* possible to detect disease progression via changes in CAL until a significant loss of attachment (2mm or greater) has occurred (1, 8). The sensitivity of measuring disease progression is low. Also, the time of detection of disease progression (2 mm CAL change) is temporally removed from the actual bone-destructive event (13). Loss of CAL represents a cumulative historical measure of periodontal disease and is not a measure of current, active disease state (14, 15). Connective tissue attachment loss can be caused by non-bacterial inflammatory sources such as trauma and therefore attachment loss by itself does not constitute evidence of a microbial insult (7, 16). Given those caveats, PD measure may be more representative of the current active inflammatory or microbial burden than CAL measure *if* CAL loss is present

concurrently. It is important to note that CAL can change without a change in PD and vice versa (9).

Lastly, bleeding on probing (BOP) is a measure of gingival inflammation that is often used in conjunction with CAL or PD (14). Normal, uninflamed gingiva should not bleed upon gentle, non-invasive probing. The absence of BOP indicates that a site with increased PD and CAL is unlikely to progress to a more advanced disease state, with an excellent negative predictive value of 98% (8).

In summary, measuring periodontitis has many complexities that can lead to erroneous assessment of the extent and severity of disease during clinical studies. This can confound measures of association between the periodontal clinical status and a clearly measurable outcome such as preterm and/or low birth weight.

Known Risk Factors for Periodontitis

Prevalence of Periodontitis

Within individuals aged 30-79 in the United States alone, the prevalence of adult chronic periodontitis ranged from 37.7 to 52.8% by state, with a mean of 45.1%. This data was calculated from the National Health and Nutrition Education Study (NHANES) 2009–2014 full-mouth, six-site-per-tooth gold-standard periodontal surveillance protocol within adults who had ≥ 2 interproximal sites with CAL ≥ 3 mm *and* ≥ 2 interproximal sites with PD ≥ 4 mm

or one site with PD ≥ 5 mm and no health conditions requiring antibiotic prophylaxis before periodontal probing. Prevalence of *severe* periodontitis, defined by CAL ≥5 mm in more than 30% of the sites that are measured, ranged from 7.27 to 10.26% by state (mean 8.9%) (17).

Microbiology

The importance of the both host immunity and the oral microbiome in initiation and progression of periodontitis can be shown through experiments utilizing germ-free mice. Germ-free mice, which lack a microbiome and consequently have an under-developed immune system, do not experience destruction of the alveolar bone as a consequence of aging (18, 19). Conventional specific-pathogen-free rodents, with a diverse oral microbiota, do lose alveolar bone as they age. Co-caging germ free animals with specific-pathogen-free mice leads to alveolar bone loss in the formerly germ-free mice (18, 19).

The tooth, coated in a film of salivary and serum proteins, provides an ideal attachment surface for long-term biofilm formation (1). When a symbiotic microbiome is present, a healthy state of homeostasis between bacteria and host immunity is maintained. When the relative proportions and diversity of microbes found in the healthy state is perturbed, the composition of the microbial community changes to a state of dysbiosis, where certain species thrive more than others (20). The dysbiosis hypothesis can be applied to periodontitis. In

this context, it could be proposed that certain strict periodontal anaerobes, found at low abundance in the normal, healthy oral microbiome, overgrow and displace the normal microbial species (18, 21-23). The dysbiotic microbiome then triggers a damaging host inflammatory response, and can also produce bacterial by-products that are directly pathogenic (24). Dysbiotic microbiomes are hypothesized to alter normal homeostatic host-microbiome communication in susceptible individuals (25).

Identifying the key species involved in oral health as compared to periodontitis poses several unique challenges, some arising from the fact that the mouth is an open system (25). The composition of microorganisms in the oral cavity can vary greatly between individuals (1). The relative importance of individual factors such as diet and host genetics in determining the composition of the oral microbiome is still debated (1, 25, 26). The community of bacteria that thrives above the gum line (supragingival) is different from the community that grows below the gum line on the root (subgingival). Nor do structures in the mouth such as the tongue and hard palate have identical microbiomes (25, 27). In the oral cavity, small variations in sampling site can make a big difference in species identified (1). Even though the individual species may vary highly, the metabolic activity of communities found in oral health are relatively stable and different from those found in periodontitis (28).

P. gingivalis, Tannerella forsythia and *Treponema denticola* are classically associated with deep periodontal pockets (23, 29, 30). *Fusobacterium*

nucleatum is another bacterium associated with deepening of periodontal pockets and disease progression. *P. gingivalis*, *T. denticola,* and *F. nucleatum* can induce lymphocyte suppression (13, 31). All these species can also be found, albeit at lower numbers, in periodontally healthy individuals (29, 32, 33). They thus behave more like commensals or opportunistic pathogens rather than true pathogens because they possess opportunistic traits when overgrowing within the community. The shifting microbial community as a whole therefore may be considered the etiological agent of periodontitis instead of a single species (34).

Most periodontal microorganisms associated with disease or disease progression were originally identified by culture techniques. Newer molecular studies employing techniques such as 16s rRNA sequencing, high-throughput next generation sequencing, and bioinformatics have confirmed and expanded the understanding of the periodontal and oral microbiome (24, 25, 35). Culture-independent molecular techniques have identified many previously unrecognized periodontitis-associated organisms. These include the phylum Synergistetes and genera *Spirochetes*, *Filifactor* and *Dialister* (24, 34, 36). Newly identified periodontitis-associated species and genera also include but are not limited to *Anaeroglobus geminatus, Eubacterium saphenum, Filifactor alocis, Porphyromonas endontalis*, *Candidatus, Saccharibacteria*, *Prevotella denticola* (24, 37-39).

Of course, bacteria are not the only microorganisms that reside in the oral cavity. For example, members of the Archaea domain, fungi, protozoans, and viruses also live within the oral microbiome (1, 25). Herpes viruses and Epstein-Barr virus in particular may be involved in the proliferation of B cells in periodontitis lesions (13).

Despite the extreme inter-patient diversity of species within the periodontal pocket, the overall metabolic capability of the community appears to be the critical factor in sustaining the microbial community in health versus disease. Human oral microbial communities undergo conserved changes in metabolism during disease. (28). The metabolic capability or profile of diseased microbiomes across individuals strongly resemble one another, even though the individual species present can vary greatly. The metabolic profile thus demonstrates conserved community gene expression. Overall enzyme expression was also conserved across disease-associated communities despite variance in species present. This suggests that many different organisms are capable of filling the same conserved metabolic niches (28).

However, the microbiota of the oral biofilm (dental and periodontal plaque biofilm) is not sufficient to induce measurable periodontal disease pathology by itself (1, 40, 41). The microbiota of the oral biofilm drives a chronic host inflammatory process that results in the characteristic loss of clinical attachment, destruction of bone support, and subsequent tooth loss (1). Thus, host factors,

notably those impacting the host immune system, also play a role in development and progression of periodontitis.

Host Factors

Immune dysregulation associated with a dysbiotic microbiome in periodontitis can manifest as an exaggerated inflammatory response against the microbial community of the periodontal pocket. This response is characterized by an inappropriate type of immune response (cellular vs. humoral) to clear a particular pathogen, inefficient clearance of specific microorganisms, failure to resolve inflammation, or a combination of all of the above (40). As a consequence of these conditions chronic gingival inflammation in susceptible individuals leads to the clinical manifestations of periodontitis.

The risk of developing periodontitis is not the same for all individuals (42-44). Hispanic, non-Hispanic black, or non-Hispanic Asian American ethnicity, age greater than 65, male sex, and uncontrolled diabetes mellitus increase the risk of developing adult chronic periodontitis (17, 45-49). Smoking and low socioeconomic status (defined by poverty or education) are conditions that can increase risk of developing adult chronic periodontitis.

While the risk of developing periodontitis in humans derives in part from habits and the microbial environment, periodontitis has a significant genetic component (43, 44). Monozygotic twins are more similar for all clinical measures of adult periodontitis than dizygotic twins. Based on twin studies, adult

periodontitis is estimated to have an approximately 50% heritability after adjusting for behavioral variables such as smoking (50). Probing depth, attachment loss, and plaque all showed a significant genetic component in twin studies (51). While adult periodontitis is estimated to have a significant heritability component, no evidence has been detected for heritability of gingivitis (50).

A few single gene disorders affecting immune cell function are known to be associated with severe, and often early-onset, periodontitis. For example, aggressive periodontitis has been associated with a number of syndromes involving decreased neutrophil number or function (44). Papillon-Lefèvre or leukocyte adhesion deficiency syndromes (LAD) are just a couple of examples (44, 52, 53).

The majority of cases of periodontitis are not tied to single gene or Mendelian disorders. Despite strong and clear evidence of heritability, genome-wide association studies have only found weak associations between genes controlling immune function and periodontitis (40). This implies that periodontitis is usually a manifestation of complex, multi-gene interactions, with each gene contributing a small amount of overall risk of developing periodontitis (54). Periodontitis is thus both polymicrobial and polygenetic (2).

Suggested polymorphisms associated with periodontitis include upstream, downstream, and within gene single nucleotide polymorphisms (SNPs) of the interleukin 1(IL-1) family, COX2, interleukin 10 (IL-10), DEFB1, and the S100A8

subunit of calprotectin (2, 55-59). The IL-1 family, COX2, and IL-10 have known roles in the inflammatory response. DEFB1 encodes an antimicrobial peptide, and calprotectin is antimicrobial through sequestration of essential ions. While these polymorphisms in genes or gene regulatory sequences significantly correlate immune function genes with periodontitis, the clinical relevance of these correlations remains poorly understood.

The key role of the immune response in the pathogenesis of periodontitis is demonstrated in animal models where immunocompetent mice lose more alveolar bone in response to colonization with a periodontal pathogen (*Porphyromonas gingivalis*) than immunodeficient mice (60). What is less clear is which components of the immune response contribute to the etiology of periodontitis. In addition to neutrophils as discussed above, autoimmune B cells, natural killer T cells, activated natural killer cells, and $CD4^+$ T cells have all been proposed as agents of host-mediated inflammation and bone loss in periodontitis (13, 32, 44, 61-73). Perhaps different individuals have different specific conditions or factors leading to dysregulation of immunity, but the underlying thread connecting all periodontitis susceptible individuals is dysregulation of host inflammation in response to an altered microbiota.

Societal Impact of Periodontal Disease

The global economic impact of oral diseases was estimated at 442 billion USD (74) in 2010, with direct treatment costs estimated at USD 298 billion. This

corresponds to 4.6% of the global health expenditure. This estimate includes dental caries (cavities) and oral cancer (75). In the United States fewer adults are covered by dental insurance than medical insurance, and for those who have dental insurance, coverage tends to be less comprehensive than medical coverage (76). Of the 92-114 billion dollars spent on dental visits in the United States in 2014, 44% were paid out-of-pocket, the highest percentage of any type of out-of-pocket expenditure for personal health care (77, 78). Periodontal procedures were estimated at 2.5% of all dental procedures in 1999 (79). The percentage of adults aged 18-64 who were unable to receive necessary dental care due to “cost” ranged between 9.3-12.6% in 1999 to 2014 (76).

Many studies have reported an association between lower quality of life and both periodontitis and gingivitis, with quality of life deceasing as disease severity increased (80-83). Individuals with periodontal disease, including severe chronic periodontitis, report more pain, more emotional stress (fear and anxiety), and greater insecurity in general. Individuals with periodontal disease report a lower functional capacity, such as speech impediments or being unable to finish meals. They may also experience social limitations or feelings of embarrassment over the state of their oral health (75, 84, 85). These factors may interact with one another, for example, a person with difficulty finishing meals may opt out of social situations that involve food. A small study found that individuals who were having difficulty accepting tooth loss were more likely to experience lack of confidence and more likely to feel inhibited by loss of teeth than individuals who

accepted their tooth loss (86). Unfortunately, current quality of life studies are mostly associative studies focused on generating mathematically testable quantitative data by the use of “yes/no” and “on a scale of” questionnaires. These studies may be lacking nuance that would be better addressed by a qualitative methodology such as key informant interviews.

Lastly, periodontal disease has been associated with several systemic conditions such as cardiovascular and pulmonary disease, rheumatoid arthritis, stroke, pneumonia, Alzheimer’s disease, and as previously mentioned diabetes mellitus (27, 87, 88). Periodontal disease, and especially inflammation associated with periodontal disease, has the potential to lead to an increased risk of developing many other debilitating disorders such as APOs.

Adverse Pregnancy Outcomes

Measuring Adverse Pregnancy Outcomes (APOs)

Pre-Birth Outcomes

Chorioamnionitis is the inflammation of the fetal membranes (chorion and/or amnion) of the placenta, often due to bacterial invasion. It is diagnosed based on maternal fever in the absence of maternal respiratory or urogenital infection and the presence of at least two of the following: maternal leukocytosis, maternal tachycardia, or fetal tachycardia (89).

Preeclampsia is a hypertensive disorder defined as elevated gestational blood pressure, such as that greater than 140/90, usually on at least two

separate occasions, and the presence of proteinuria (90, 91). Urinary tract infections have also been associated with preeclampsia (92). Preeclampsia affects only 5-10% of pregnancies but contributes significantly to maternal and perinatal mortality and morbidity (90, 91).

Chorioamnionitis and preeclampsia are both associated with adverse birth outcomes.

Birth Outcomes

Preterm birth (PTB) in humans is defined as birth before or at 37 weeks of gestation, with extreme prematurity being defined as at or below 32 weeks of gestation. Survival at less than 24 weeks gestation occurs only in very rare situations (93). In the United States in 2015, the prevalence of PTB was 9.63%. PTB increased between 2007 and 2015 among non-Hispanic black and Hispanic women (94).

Low birth weight (LBW) is defined in humans as birth weight below 2500 g and very low birth weight at less than 1500 g (95). Newborn survival at less than 500 g of weight is rare (93). The prevalence of LBW in the United States in 2015 was 8.07% (94). Since both conditions are related, they are often referred to together in the literature as preterm low birth weight (PLBW). PLBW in many cases is a consequence of preterm labor (PTL) and/or premature rupture of membranes (PROM) (96). PTL can be associated with subclinical infection and intrauterine inflammatory response (97).

Known Risk Factors for Adverse Pregnancy Outcomes

Host Factors

Known host risk factors for APOs include: maternal age greater than 34 years or less than 17 years, low socioeconomic status, inadequate prenatal care such as missed prenatal ultrasound appointments, recreational drug abuse, alcohol use, smoking or other tobacco use, hypertension, diabetes, multiple pregnancies, and previous incidence of PLBW (15, 98). These risk factors should not be assumed to be entirely independent of one another.

Some racial and ethnic groups, such as non-Hispanic black women, are especially susceptible to LBW. Whether the increased risk of APOs in certain ethnicities is due to an increased prevalence of other associated risk factors, such as low socioeconomic status, in these groups is still debated. Problems related to PTB or LBW were the primary cause of deaths among black infants born in the United States (98). In 2013 the Infant Mortality Rate (IMR), defined as deaths within the first year of life per 1,000 live births, for blacks was 11.11 as compared to approximately 5 for whites and Hispanics. American Indian or Native Alaskans had an Infant Mortality rate of 7.61 (93). The risk of APOs, and/or infant mortality following PLBW, is therefore not equal for every pregnancy, even within the same woman.

Some of the difference in risk of APOs between racial or ethnic groups may be explained by differences in the vaginal microbiome. For example, white

women were more likely to have a vaginal flora dominated by *Lactobacillus* than black women, and black women were more likely to have microbiomes dominated by strict anaerobes (99). The increased prevalence of vaginal anaerobes may also correspond with the higher rate of bacterial vaginosis in black versus white women (51 versus 23%, respectively) (100). It thus behooves us to evaluate not only maternal host factors but also maternal microbial factors in the pathogenesis of PLBW.

Microbial Factors

Intrauterine infections and infections of the genitourinary tract have been associated with PTB and LBW (98, 101, 102). There is clear evidence that bacterial vaginosis (BV) is an independent risk factor for PLBW, PROM, PTL, and chorioamnionitis (98, 101, 102). BV results from an overgrowth of organisms that replace the normal vaginal microbiome (98). The normal vaginal community of *Lactobacillus* at pH 4.5 is displaced and replaced by organisms surviving at higher pH such as *Gardnerella vaginalis, Mycoplamsa mainis*, *Mycoplasma hominis*, and *Bacteriodes* spp. (98, 101). *Gardnerella vaginalis* and *Bacteroides* spp. in particular have been associated with both BV and PTL (103). *Bacteroides* spp. have also been associated with PLBW and PROM (103, 104). Despite the name, BV does not necessarily involve inflammation of the vaginal walls or cervix but can be diagnosed by presence of vaginal epithelial cells covered in adherent bacteria (98). An estimated 50% of women with BV are

asymptomatic yet still at an increased risk of PTB or LBW (98). The odds ratio of preterm delivery among women with BV has been demonstrated to be between 1.4 to 2.8, to as high as 6.9 in a limited number of studies (98, 101, 102).

Vaginal infections with species not normally associated with BV have also been reported in association with APOs. *Escherichia coli*, *Klebsiella* spp., *Haemophilus* spp., *Ureaplasma urealyticum*, and *S. aureus* in the vaginal flora have been associated with PTL (103, 104). *Klebsiella* spp., *Haemophilus* spp., and *Trichomonas vaginalis* were associated with PROM (103, 104). Group B streptococci in the vagina or urine of pregnant women has been associated with PTB and PROM (105-107).

Of course, host maternal immunity may influence the ability of key pathogenic microorganisms to colonize the female reproductive tract. Therefore, the host microbiome and host immunity should not be considered as separate from one another. However, many cases of PTB or LBW are not associated with any known risk factors, maternal or microbial. This suggests that there are additional risk factors that are not currently understood (98). Failure to account for the known associated risk factors is also unfortunately a problem in the design of many studies attempting to identify novel risk factors, such as periodontitis, for PLBW (108, 109).

Societal Impact of Adverse Pregnancy Outcomes

The Infant Mortality Rate (IMR) as shown in Table 1 demonstrates the high personal cost that PTB and LBW impose on families.

Table 1. 2013 Infant Mortality Rate (Deaths per 1000 Live Births) in the United States (93)

Gestational Weeks	IMR	Weight at Delivery	IMR
Normal Term Birth 37-41 weeks	2.19	Full Term Weight ≥ 2500 grams	2.05
Preterm Birth < 37 weeks	34.79	Low Birth Weight < 2500 grams	50.26
Very Preterm Birth < 32 weeks	163.71	Very Low Birth Weight < 1500 grams	219.56

PTB and LBW have consequences beyond infancy. For example, gestational age at delivery strongly determines the risk of impaired neurodevelopment. Fifty-two percent of infants born at 24-28 weeks, 24% of those born at 28–31 weeks, and 5% of those born at 32–36 weeks were estimated to have some degree of neurodevelopmental deficit (110). There is also evidence that PTB or LBW increase the risk of diabetes mellitus in adulthood (95). Individuals that were born preterm and had very low birth weight (below 1500 grams) were at higher risk of having insulin resistance, glucose intolerance and higher blood pressure at 18 to 27 years of age than those born at term (95).

Children born prematurely had increased rates of chronic coughing, wheezing, asthma, and asthma-like symptoms from infancy through their school years. These conditions lead to an increased risk of childhood hospitalization for breathing disorders in those born prematurely (111-113). To give one final example, adults between the ages of 18 and 27 who were born premature and with very low birth weight have less bone mineral density than term birth and normal weight peers (114).

Given the prevalence of PTB and LBW and their cost to society, families, and individuals, identifying strategies to reduce or eliminate APOs is a critical public health priority. We will now evaluate the connection between oral health and PLBW to support the notion that preventative treatment of periodontitis may lead to a reduction in devastating APOs.

Pregnancy Gingivitis versus Periodontitis in Pregnancy

To understand the literature connecting oral health to APOs it is important to distinguish pregnancy gingivitis from periodontitis. Pregnant women are particularly susceptible to gingival inflammation and gingival bleeding, and thus subsequent bacteremia (115-117). However, pregnancy gingivitis is very different from periodontitis in pregnancy, and the two should not be confused.

Pregnancy gingivitis is an extremely common, reversible condition of gingival inflammation has been characterized as “non-specific, vascularizing, and proliferative inflammation with large amounts of infiltrated inflammatory cells” (118). Women with healthy gingiva and free of dental plaque are unlikely to develop pregnancy gingivitis. In pregnancy gingivitis, the gingiva may become dark red and bleed easily. Deeper probing depths may not be indicative of true periodontal destruction in pregnant women. Swelling and gingival inflammation without bone loss may account for increased probing depth. Pregnancy gingivitis is associated with the high levels of steroid hormones (estrogens). Feeding on these hormones, microbial species such as *Prevotella intermedia* bloom within the microbial community (118-124). When good oral hygiene practices are implemented or maintained, pregnancy gingivitis resolves itself within a few months of birth with no permanent changes in CAL (118, 121, 122, 124). Pregnancy gingivitis is not considered a risk factor for APOs.

Periodontitis is also associated with a shift in the relative composition of the oral microbiome, but the shift is unrelated to pregnancy status or pregnancy hormones. This is an important distinction to make, as gingivitis and periodontitis are both forms of periodontal disease. The difference in dominant species present in pregnancy gingivitis versus periodontitis in pregnancy may explain why pregnancy gingivitis is not associated with APOs, even though an increased risk of bacteremia would be present in both conditions (117-121, 123, 124). Unfortunately, some studies that have been included in meta-analysis measuring the association between periodontitis and APOs measure an association between *gingivitis* and APOs (125).

Periodontitis Definition Affects Studies of Adverse Pregnancy Outcomes

One problem with interpreting any association between periodontitis and pregnancy outcomes is that the cut-off point of periodontitis severity and extent utilized to separate a “case” from a “control” is not consistent across many studies of APOs (6, 126). One particularly clever analysis demonstrated that varying case definitions may change the significance of the association between periodontitis and APOs (126). This study utilized 14 different case definitions of periodontitis on the same data set and discovered that only 6 of the 14 tested definitions yielded a significant association between periodontitis and various APOs. The robustness and validity of any reported association between

periodontitis and proposed outcomes, including but not limited to APOs, is therefore critically dependent on the criteria used to define a periodontitis patient and study inclusion criteria.

Failure to control for confounders can also bias study results. Increasing age, diabetes mellitus, low socioeconomic status, non-Hispanic black ethnicity, and tobacco use are risk factors that are associated with both periodontitis and APOs (15, 45, 46, 98). Failure to control for these factors could lead to reporting of spurious associations between periodontitis and APOs (108, 109, 127).

Association of Periodontitis and Adverse Pregnancy Outcomes

To effectively reduce the cost of APOs to families and society, novel therapeutic interventions must be designed that can fill the current gap in preventive care. To design these future interventions, the risk factors and etiological agents of APOs must be understood. While many risk factors for APOs are known, some clearly are not well established. Based on observational and epidemiological studies, periodontitis has been proposed as one of those missing risk factors. Scaling and Root Planing (SRP) trials were an attempt to develop a novel therapeutic intervention to prevent APOs, but they failed. These SRP studies nonetheless have progressed our understanding of the mechanism that connects periodontitis with APOs and can lead us to design more effective therapies to prevent APOs in women with periodontitis.

Prevalence of Periodontitis in Pregnant Women

Periodontitis prevalence during pregnancy is important in determining whether the risk of APOs due to periodontitis has a significant impact on public health. Data from the National Health and Nutrition Education Study (NHANES) has been used to estimate the prevalence of periodontitis in United States population. NHANES, until 2001, measured only two periodontal sites on each tooth (mesial and mid-buccal) of two randomly selected quadrants. Since not all 6 sites around a tooth were measured, it is likely that the true prevalence of periodontitis was underestimated (45). Some have suggested partial-mouth examinations may underestimate actual periodontitis prevalence rates by as much as 50 percent (128). NHANES began using 6 sites per tooth, full mouth evaluations of periodontitis in 2009 (129). Unfortunately, 2004 is the last reported time period wherein the prevalence in periodontitis in pregnant women specifically was evaluated with NHANES data. Periodontitis was defined in NHANES for the period between 1999 and 2004 as one periodontal site with ≥3 mm of clinical attachment loss (CAL) and ≥4 mm of probing depth (PD) (130).

Within the NHANES data set 1999-2004 using the above definition, the prevalence of periodontitis in pregnancy was higher with increasing maternal age. The prevalence of periodontitis during pregnancy was 6.7% in women aged 35-44 versus 2-3% in women aged 15-34 of all ethnicities and all education levels. The prevalence of periodontitis during pregnancy also increased with lower levels of education, as 5.7% of women with below-high-school education

level versus only 2-3% of women with a high school diploma or advanced degree were affected when all ethnicities and all ages are considered.

Determining something as seemingly simple as prevalence can be dependent on definition of periodontitis. The following table shows the differences between two studies which utilized different definitions of periodontitis to evaluate the prevalence of periodontitis in pregnant women by ethnicity (130, 131). The study with a broad definition of periodontitis (131) found a higher prevalence than the study with a narrower definition (130), as would be expected (Table 2). It is unlikely that the prevalence of periodontitis in pregnant women truly significantly decreased between 1994 and 2004.

Table 2. Prevalence of Periodontitis in Pregnant Women in NHANES Data Set varies by Definition of Periodontitis

	Xiong *et al.* 2006 (131)	**Azofeifa *et al.* 2016 (130)**
	NHANES data 1988-1994 n=268	NHANES data 1999-2004 n=772
Periodontal disease definition	at least one site with CAL ***or*** PD ≥4mm	at least one periodontal site with ≥3 mm CAL ***and*** ≥4 mm of PD
Non-Hispanic Whites	10.7%	2.0%
Non-Hispanic Blacks	29.1%	2.2%
Mexican-Americans	20.6%	9.3%

Observational Studies of Periodontitis and APOs

Observational studies include case-control, cross-sectional, longitudinal, and cohort studies. Due to the heterogeneity in the definition of periodontitis used to assign women to case or control groups, there are few meta-analysis of observational studies testing the association between periodontitis and APOs (108, 109). One meta-analysis of 15 observational studies found that periodontitis significantly increased the risk of PTB (odds ratio (OR) 2.73, 95% CI 2.06-3.6, $p<0.0001$) and LBW (OR 1.5, 95% CI 1.26-1.79, $p = 0.001$) (132). A systematic review concluded that there were twice the number of case-control and cohort studies that detected an association between periodontitis and APOs as compared to studies that did not detect an association (108). Epidemiological studies also find an association between periodontitis and APOs (133). Therefore, we can unequivocally conclude that a significant fraction of women experiencing APOs (~2.0 to 9.3%) are also affected by periodontitis, with the caveat that periodontitis case definition influences the extent of association between periodontitis and APOs. This fact led to the hypothesis that treating periodontitis would reduce APOs.

Scaling and Root Planing to Prevent APOs

Several large, randomized control treatment trials (RCTs) using scaling and root planing (SRP) were thus designed to test whether a periodontal therapeutic intervention could reduce the risk of APOs in women with

periodontitis. Two NIDCR funded trials, OPT and MOTOR, independently determined that second trimester SRP therapy did not either increase or reduce the risk of APOs in women with periodontitis (134).

A meta-analysis of current SRP studies used Cochrane's risk of bias tool to assess methodological quality of studies that were evaluated. Studies were separated into "high" and "low" quality studies based on risk of bias, such as selection bias or attrition bias (125). This analysis found that SRP treatment did not reduce the risk of PTB (OR 0.93, 95% CI 0.79-1.10[1]) (125). Interestingly, low quality trials suggested that treatment was beneficial to prevent PTB (OR 0.52, 95% CI 0.38-0.72) whereas high quality trials suggested no effect of treatment (OR 1.15, 95% CI 0.95-1.40). The same difference in high vs low quality studies can be seen in regards to the effects of SRP treatment on LBW. Low quality studies found a benefit of SRP treatment on reducing the risk of LBW (OR 0.44, 95% CI 0.30-0.66) whereas high quality studies did not (OR 1.07, 95% CI 0.85-1.36) (134-138). Combining low and high quality studies demonstrated that SRP treatment had no effect on LBW (OR 0.85, 95% CI 0.70-1.04) (125). It has been observed that smaller studies, which are more vulnerable to the effects of bias, are more likely than larger studies to show a positive effect of treatment of periodontitis on PLBW (139).

[1] Odds ratio (OR) with a confidence interval that spans the value 1 are non-significant ($p > 0.05$). Odds ratio with a confidence interval where both values are less than 1 or both values are greater than 1 are significant ($p \leq 0.05$).

On an interesting note, leaving aside quality of evidence, 5 studies within this meta-analysis looked at both CAL and PD, 5 studies evaluated only CAL, and 1 study used PD only to define disease severity. Two studies additionally considered BOP (125). One study was evaluating patients experiencing gingivitis, not periodontitis (140). These details are important in light of the knowledge that heterogeneity in how cases and controls are defined influences the extent of association between APOs and periodontitis.

Additional meta-analysis of SRP treatment agreed that in high-quality studies SRP does not reduce the risk of PTB (OR 0.81, 95% CI 0.64-1.02), (OR 1.082, 95% CI 0.891-1.314), (RR 0.88, 95% CI 0.72-1.09) or the risk of LBW (OR 0.72, 95% CI 0.48-1.07), (OR 1.181, 95% CI 0.960-1.452), (RR 0.78, 95% CI 0.53-1.17), just to list those RCT that evaluated risk of bias in included trials (139, 141, 142). Collectively we can therefore conclude that in high-quality, low risk of bias studies, SRP does not reduce the risk of APOs. These findings prompted researchers to question the mechanism behind the association between periodontitis and increased risk of APOs found in observational studies (108, 109, 132, 133).

A Potential Explanation for the Failure of SRP Therapy to Prevent APOs

Some critics maintain that failure of treatment trials indicate that there is no association between periodontitis and APOs. This criticism applies a faulty

logic because it assumes *a priori* that microorganisms residing only in the periodontal pocket are the sole contributors to APOs. However, there are alternative explanations for the lack of reduction in APOs with periodontal treatment. For example, SRP could have been either the wrong treatment to resolve the issue, or SRP could have been delivered too late in pregnancy to prevent APOs (127). One meta-review on SRP and APOs noted that the selected studies, except two, used “mild periodontitis” definition as inclusion criteria (125). This raises the additional possibility that SRP may not be as effective in treating APOs in women with mild cases of periodontitis as it could have been if only severe cases of periodontitis were treated. Including mild periodontitis cases in treatment studies may have diluted the potential effect of treatment on APOs.

Another possibility is that while SRP changes the composition of the periodontal microbiota by mechanically disrupting the biofilm within the periodontal pocket, SRP has no impact on potential hematogenous spread of periodontal pathogens to the placenta occurring *prior* to SRP treatment. Notably, for safety reasons, all SRP trials were performed during second trimester. Since gingival inflammation during pregnancy increases the likelihood of transient bacteremia, it is plausible that periodontal pathogens seed the placental tissue and either the pathogens themselves or the inflammatory immune response to them may impair the developing fetus (115-117). This hypothesis is corroborated by recent studies showing that the placenta harbors a unique, low abundance

microbiome that is similar to the oral microbiome (143). This finding is in stark contrast with previous assumptions that deemed the placenta an essentially sterile environment (144-146). The composition of the newly discovered placental microbiome changes in association with various APOs (143, 145, 147).

Periodontitis-associated microorganisms, like *P. gingivalis,* seeding the placenta could have a direct effect on placental viability and function through bacterial virulence factors (direct theory). The maternal inflammatory immune response may influence the ability of periodontal pathogens to colonize the placenta. Alternatively, the maternal inflammatory immune response against periodontal pathogens and/or dysbiotic placental microbiome may compromise nutrient transfer to the fetus and increase the risk of APOs (indirect theory). Placental colonization with periodontal microorganisms, therefore, appears to be a plausible mechanism by which periodontitis increases the risk of APOs.

This thesis will present evidence obtained from a mouse model of periodontitis to argue that periodontitis increases the risk of APOs because of the spread of periodontal pathogens to the fetoplacental unit.

Periodontal Pathogens Implicated in Adverse Pregnancy Outcomes

Porphyromonas gingivalis and *Fusobacterium nucleatum* are two anaerobic species commonly associated with both periodontitis and APOs in

humans (23, 26, 30). Found in relatively low abundance compared to all other microorganisms, *P. gingivalis* can have large pathogenic effects by changing the local nutrient foundation of the microbial community (18, 148, 149). *P. gingivalis* therefore meets the definition of a keystone pathogen. Changes in the nutrient base destabilize the relative proportions of the normal microbial community. The resulting dysbiotic microbiome alters the host-microbiome homeostasis, leading to a more pro-inflammatory response (148). *P. gingivalis* is unable to induce bone destruction when inoculated by itself into germ-free animals, whereas in conventional or specific pathogen free (SPF) murine colonies *P. gingivalis* drives alveolar bone destruction. This further demonstrates the need for a microbial community to elicit pathogenic effects as *P. gingivalis* increases the overall community virulence by enabling proportional shifts in the composition of a previously friendly microbiome (2, 150).

There is evidence for a role of *P. gingivalis* in not only periodontitis but also in APOs. *P. gingivalis* can be detected in placentas of fetal growth restricted BALB/c mouse fetuses after subcutaneous inoculation with *P. gingivalis* (151, 152). Oral inoculation of BALB/c mice with *P. gingivalis* plus other periodontal pathogens leads to upregulation of toll-like receptor 4 (TLR4) mRNA in placental labyrinth tissue. The TLR4 pathway is known to be involved in the pathogenesis of some cases of PTB (153). Changes in TLR regulation demonstrate that *P. gingivalis* can induce a host inflammatory immune response in placental tissues.

F. nucleatum has also been linked to APOs in both mice and humans (154, 155). *F. nucleatum* interacts with a diverse range of oral species and positively influences the biomass of other Gram negative anaerobes (33). Parenteral inoculation with human plaque samples into pregnant mice results in preferential seeding of *F. nucleatum* to the placentas (115) possibly through the action of fap2 (156) and/or fadA adhesion proteins (157, 158). *F. nucleatum* can lead to murine fetal death through stimulation of TLR4 and not through direct bacterial toxicity (159). These findings suggest that *P. gingivalis* and *F. nucleatum* elicit a detrimental inflammatory response or that the direct virulence of the microorganisms can be responsible for APOs.

Evidence of Periodontal Pathogens in the Placenta

Species-specific PCR-based Detection of *P. gingivalis* and *F. nucleatum* in Placentas Associated with APOs

Species-specific conventional PCR- or quantitative (qPCR)-based studies differ from DNA sequencing studies in that they start with an *a priori* defined set of potential species to evaluate. This tends to bias species-specific studies towards the detection of well-established cultivatable periodontal pathogens over lesser characterized uncultivatable species (160). Conventional PCR is not as efficient at quantitating microorganisms as DNA sequencing or qPCR. Nonetheless, conventional PCR can provide important information about the

presence but not the abundance of various organisms in conditions of disease versus health.

When analyzed with conventional PCR, placental tissue samples in women with PLBW infants have yielded higher species counts than controls (160). Specifically, *F. nucleatum* was detected in 94% of placentas from mothers with periodontitis and PLBW as compared to 36.4% of full-term placentas from mothers without periodontitis. *F. nucleatum* has also been detected in the placenta of a stillborn infant from a mother who experienced excessive gingival bleeding during pregnancy. This *F. nucleatum* strain was genetically identical to that found in the mother's oral cavity (155). Nested PCR has revealed that *F. nucleatum* was more prevalent in mothers with periodontitis and PTB ($p = 0.033$) (160).

Preeclampsia has also been associated with maternal periodontitis (90). Pooled samples from maternal and fetal placental tissues of preeclampsia patients revealed the presence of *Actinobacillus actinomycetemcomitans, F. nucleatum*, and *P. intermedia* (91). *P. gingivalis, F. nucleatum, Tannerella forsythensis*, and *Treponema denticola* were detected in significantly higher numbers in preeclampsia patients than in control subjects when detected with qPCR (91). In another study, conventional PCR placental samples from preeclampsia patients revealed presence of *Bacillus, Variovorax, Prevotella shahii, Porphyromonas*, *Dialister*, and *Lactobacillus*. Importantly, this study did not detect the presence of bacterial DNA in maternal blood in any patient (161).

This suggests that oral microorganisms can enter the bloodstream infrequently or at below detectable levels after manipulation of the gingiva with brushing or even after chewing food, or possibly even shielded within a few phagocytes (150). However, upon reaching the placenta through the blood oral pathogens may colonize and grow locally to detectable levels.

Species-specific Immunohistochemistry and Immunofluorescence in Placentas Associated with APOs.

Placental sections from chorioamnionitis-affected human placentas associated with PTB showed 30% more intense immunostaining for *P. gingivalis* antigens than normal placental controls. Staining was observed in placental synctiotrophoblasts, chorionic trophoblasts, decidual cells, amniotic epithelial cells, and vascular cells. The most commonly stained areas regardless of case or control status were trophoblasts, decidual cells, and vasculature cells (89).

A second study reported that *P. gingivalis* in the placenta was associated with shorter gestations and delivery by caesarean section, but not with chorioamnionitis or preeclampsia (162). *P. gingivalis* antigen were only detected in the villous mesenchyme of PTB birth placentas but not of full term controls. In contrast, *P. gingivalis* antigen were detected in syncytiotrophoblasts of both preterm and full term gestation placentas (162). Notably, in this same study preeclampsia patients had *P. gingivalis* antigens only in the umbilical cord but not in the placenta.

Collectively, PCR-based and immunohistological findings suggest that at least two well established periodontal pathogens, *F. nucleatum* and *P. gingivalis*, can be detected in placentas associated with APOs in humans and mice. It is possible that *P. gingivalis* colonization of certain areas of the placenta leads to different APOs. Since there is disagreement between studies on the localization of *P. gingivalis* in the fetoplacental unit and subsequent APOs, more studies are needed to confirm the exact relationship between colonization of specific areas of the placenta and specific APOs.

Detection of increased systemic levels of interferon-gamma (IFN-γ) in animal models of APOs suggests that the maternal immune response may induce inflammation and subsequently impairs fetal growth. Presence of oral pathogens such as *P. gingivalis* in placentas associated with APOs suggests that periodontal pathogens could be inducing an IFN-γ driven maternal immune response that is detrimental to placental and fetal health. Alternatively, the changes *P. gingivalis* induces in the nutrient base leading to a more pathogenic oral or placental microbiome may be driving increased IFN-γ production. Certain subsets of $CD4^+$ T cells are major, but not sole, producers of IFN-γ. Additionally, the presence of *P. gingivalis* in the placenta could act as a local re-stimulator of T cells, leading to local production of IFN-γ in the placenta by $CD4^+$ T cells. However, IFN-γ produced by $CD4^+$ T cells responding to oral pathogens has not yet been definitively established as a contributor to fetal growth restriction.

Evidence of $CD4^+$ T cell Role in Periodontitis and Adverse Pregnancy Outcomes

The type of defense mounted against a given pathogen depends in part on immune response characteristics of the host. For example, the T cell response to the obligate intracellular bacterium *Mycobacterium leprae* in humans can lead to either tuberculoid leprosy (Th1-driven) or lepromatous leprosy (Th2-driven). The Th2 type response leads to the inability to contain the infection and to a more severe pathology (163). Similarly, there is evidence that the type of Th response to periodontal pathogens such as *P. gingivalis* within a given individual may lead to host resistance or host susceptibility to alveolar bone destruction and APOs. Additionally, genetically-defined differences in adaptive host immunity might influence the ability of key periodontal pathogens such as *P. gingivalis* to invade and permanently colonize the host oral or placental microbiome.

Before presenting the specifics of $CD4^+$ T cells in periodontitis and APOs, it is prudent to define key $CD4^+$ T cell subsets and their roles in immunity. The number of $CD4^+$ T cell subsets has expanded over time after the characterization of novel phenotypic behavior of each subset (97). The subsets most relevant to both periodontitis and APOs today are discussed in table 3.

Table 3. Phenotypes of Effector $CD4^+$ T helper (Th) and T regulatory (Treg) Cell Subset

T cell subset	Characteristic cytokines	Role in immunity
Th1	IFN-γ	Defense against intracellular pathogens, assists macrophages
Th2	IL-4, IL-5	Immunity against extracellular pathogens, humoral immunity, helps class switched antibody production by B cells
Th17	IL-17 family (IL-17A and IL-17E)	Defense against fungi, immunity at mucosal sites, assists neutrophil recruitment
Treg	FoxP3 transcription factor	Control and dampen other immune cell responses

Evidence for a Role of $CD4^+$ T cells in Periodontitis

Numerous studies demonstrated that deletion of $CD4^+$ T cells results in resistance to alveolar bone loss (reviewed in (164)). Knockout of major histocompatibility (MHC) class II genes leads specifically to absence of $CD4^+$ T cells and results in protection against alveolar bone loss after oral *P gingivalis* colonization (73). Genetic deletion of alpha chain of T cell receptor prevents the development of both $CD4^+$ and $CD8^+$ T cells and results in a similar protective effect against bone destruction (69, 72). Mice lacking IFN-γ or IL-6 are not susceptible to bone destruction (69). Subcutaneous immunizations of C57BL/6 mice with *P. gingivalis* antigens, combined with adjuvants known to elicit a Th1 response, resulted in extensive alveolar bone destruction with periapical granulomas heavily infiltrated by osteoclasts after re-challenge with whole *P. gingivalis.* Th2-biased mice had minimal bone lesions without obvious osteoclast infiltration (165). Given the evidence that T cells can impact periodontal outcomes in mice, we looked for evidence of T cell involvement in human periodontitis.

Evidence for a Role of Th1 and Th2 cells in Periodontitis

Human studies have been contradictory as to the role of the cytokines produced by Th cells in periodontitis. Most studies have concluded Th1 cells were associated with stable periodontal lesions, whereas Th2 cells were associated with periodontal disease progression, but not all studies agree (13,

166). Problematically, these studies were done before the understanding that T helper cells constituted more subsets than just Th1 and Th2. This means that in early studies some conclusions about T cell behavior in periodontitis that were attributed to the actions of Th1 may have actually been driven by Th17 cells.

T cells in human diseased gingival tissue predominantly express CCR5 and CXCR3 chemokine receptors characteristic of Th1 cells. Ligands for these receptors were also elevated in diseased tissues (167, 168). IFN-γ-producing Th1 cells can be a source of membrane bound and soluble receptor activator of nuclear factor kappaβ ligand RANKL (169-172). *P. gingivalis* colonization can induce RANKL production by T and B cells. RANKL induces differentiation of mature osteoclasts, which are responsible for bone resorption (169, 173).

Other studies found Th2 cells to be more abundant in periodontitis lesions (174). A slightly increased Th2 response (higher Th2/Th1 ratio) was found in peripheral blood of aggressive periodontitis patients as compared to chronic periodontitis patients or healthy controls (32). Unstimulated cells isolated from chronically-inflamed tissues of patients with early-onset or advanced periodontitis mostly produced Th2 cytokines (175).

Finally, some studies have reported that $CD4^+$ Th cells isolated from inflamed gingival tissues of periodontitis patients can express both Th1 and Th2 cytokines (176, 177). This may explain the dichotomy between studies that report a dominance of Th1 cells and those that report a majority Th2 cells in periodontitis lesions.

The dominance of Th1 or Th2 cells may also depend on the type of periodontal pathogens present. *P. gingivalis* in chronic periodontitis patients is associated with a higher percentage of IFN-γ producing $CD4^+$ T cells, leading to a higher IFN-γ/IL-4 (Th2) ratio of cytokine (32). Lack of IFN-γ in IFN-γ-knockout mice protects against alveolar bone loss induced by *A. actinomycetemcomitans*, but leads to disseminated bacterial infection and death (178). In contrast, *T. forsythia*-induced alveolar bone loss in BALB/c mice was mediated by a TLR2-dependent Th2 response (179).

Evidence for a Role of Th17 and Treg cells in Periodontitis

IL-17 expression is greater in periodontitis tissues when compared to healthy control tissue (180-185). Cytokines necessary for Th17 cell differentiation are detected in inflamed periodontal tissue (40). Elevated levels of mRNA for Th17-driving cytokines like IL-1β, IL-6 and IL-21 were detected in gingival tissue of periodontitis patients compared to healthy controls (181, 186, 187). In severely inflamed periodontitis sites, macrophages producing IL-23 appear to amplify the memory Th17 response (182). Cell culture supernatants from human antigen presenting cell culture stimulated with the periodontal pathogen *P. gingivalis* induced a Th17 response (188).

Increasing numbers of $IL\text{-}17^+$ T cells in specific pathogen free rodents may be correlated with age-associated alveolar bone loss (19). The endogenous inhibitor of neutrophil adhesion (Del-1), inhibits IL-17-dependent, neutrophil-

induced pathology, and consequently inflammation-associated bone loss in mice and non-human primates (189, 190).

The role of IL17 in periodontitis is still debated because of data originating from animal models. Female C57BL/6J mice lacking the IL-17 receptor (IL-17RA^{KO}) experienced reduced serum chemokine levels and reduced neutrophil migration to bone after *P. gingivalis* inoculation (191). Female BALB/cJ IL-17RA^{KO} were defective in production of immunoglobulin G (IgG) against *P. gingivalis* and lacked essential chemokine responses. In contrast males failed to upregulate granulocyte colony-stimulating factor. (192). These two studies demonstrate then that this specific immune defect in two strains of female mice leads to increased susceptibility to alveolar bone loss.

Treg cells ($CD4^+$ $CD25^+$ $FoxP3^+$) play a critical role in modulating the production of pro-inflammatory cytokines in gingival tissues protecting against bone destruction. Tregs downregulate the immune response of other $CD4^+$ T cells via IL-10 and TGF-β (193). IL-10 also inhibits RANKL activity and deficiency of Treg cells leads to an increase in RANKL activity with increased bone alveolar destruction (194). Treg cells attenuate experimental periodontitis in *A. actinomycetemcomitans* infected C57BL/6 mice (195).

Interestingly, $FoxP3^+$ T cells were found in greater numbers in gingivitis lesions than IL-17A producing cells. This was especially true for intensely inflamed gingival tissue, and B and plasma cell-predominant gingival tissues (196-198). The association of increased Tregs and increased severity of disease

suggests not that Tregs are responsible for periodontal destruction, but that Tregs attempt to dampen the pro-inflammatory response but are ultimately unable to constrain inflammation.

Ultimately, it is likely that different T cell subsets are associated with different stages of disease, or with the difference between stable and progressive periodontal lesions. It is also likely that unique microbial communities stimulate the differentiation of naïve $CD4^+$ T cells into different subsets of effector $CD4^+$ T cells. Certain communities may drive the type of inflammation that protects from bone destruction and other communities may promote bone destruction depending on the overall cytokine milieu. Undoubtedly, different genetic make-ups may also lead to periodontitis or APOs "susceptible" and periodontitis or APOS "resistant" individuals.

Evidence for a Role of Th1 cells in APOs

There is a strong body of experimental evidence demonstrating the involvement of high levels of systemic IFN-γ in APOs. Studies showing a role of Th1 related to failure of fetoplacental implantation in humans have been mixed. Since many of these studies were performed prior to the understanding of Th17 and Treg cells, it has been suggested that the balance of Treg to Th1 cells is important rather than absolute numbers in determining whether higher Th1 cells are detrimental to fetoplacental implantation outcomes (97). Similarly, a high frequency of Th1 and low Th2 type cells is associated with spontaneous abortion,

as opposed to absolute numbers of either. Th1 type cytokines (IL-2, IFN-γ, TNFα) are up-regulated in PTL, but so are the Th2 cytokines (IL-4, IL-6) (97).

Th1-type immunity dominates in preeclampsia, but IFN-γ, which is commonly used as a measure of Th1 activity, can also be produced by other immune cells such as natural killer cells. High serum Th1-type immunity, combined with significantly lower numbers of Treg cells in women with preeclampsia, may represent a failure of immune tolerance (97).

Th1-associated cytokines have also been implicated in APOs in mice. Non-pregnant C57BL/6/J mice respond to *Leishmania major* by producing IFN-γ, and experience failure of implantation, fetal resorption, and problems with placental development. In contrast BALB/c mice have a Th2 response against *Leishmania* that is insufficient to clear the pathogen and can lead to lethality of the dam, but does not lead to fetal loss per se (199, 200). Pregnant C57BL/6J are less able clear *L. major* infection due to greater amount of systemic IL-10 leading to a more severe pathology. Administration of IL-2 or IFN-γ, or stimulation of Th1-type cytokine production through TLR ligation, leads to increased incidence of abortion in mice (97). Subcutaneous inoculation of BALB/c mice with *P. gingivalis* leads to fetal growth restriction correlated with an increase in $CD4^+$ Th1 type cytokines such as IFN-γ (151, 152).

Other animal models have also demonstrated an association between Th1-type cytokines such as IFN-γ and APOs. The placenta of rabbits is more like the one of humans than the one of mice. IFN-γ has been linked with

apoptosis of the rabbit placenta, particularly the placental trophoblasts, and reduced progesterone production (201, 202). Studies show conflicting evidence for both a detrimental role and a protective role of IFN-γ in fetal death and abortion in cattle infected with *Neospora caninum* (203-205). In rats and humans, IFN-γ has been associated with apoptosis of placental cytotrophoblasts. Apoptosis has also been shown in response to IFN-γ in primary rat placental decidual cells (206, 207). At least one study has shown that the Th1-associted cytokine TNFα, but not IFN-γ, is correlated with intrauterine inflammation in rats (208). Subcutaneous *P. gingivalis* induce low fetal weight in hamsters secondary to increased prostaglandin E2 (PGE2) and tumor necrosis factor alpha (TNFα) (209, 210).

Importantly, IFN-γ is necessary for vascular remodeling early in mouse pregnancy. Thus, too much or too little IFN-γ can both be detrimental (97). The balance of Th1 to either Th2 or Treg cells could be key to unveiling a potential role for Th1-associated cytokines in APOs.

Evidence for a Role of Th17 in APOs

The role of Th17-associated cytokines in APOs has been far less studied than the role of Th1-associated cytokines. For example, whether Th17 cells are involved in spontaneous abortion, or present in reproductive tissues as a consequence of abortion, is still debated (97). Although Th17 cells may protect the uterine cavity against extracellular microbes, it appears that the Th17

response is dampened during pregnancy (97). During pregnancy, the signs and symptoms of IL-17/Th17-driven rheumatoid arthritis are alleviated. Th17 cell numbers are also increased in the chorioamniotic membranes from PTB infants of mothers with chorioamnionitis. Th17 cells can be observed to promote inflammation at the fetomaternal interface in preterm delivery. (97). In normal pregnancy, peripheral blood shows increased Treg and decreased Th17 cells whereas in preeclampsia women show the opposite trend (97).

Ultimately, the Th17 response is currently understudied in pregnancy. The Th17 response appears to be heightened during some APOs when the ratio of Th17 to Tregs is increased. Interestingly, the Th17 response generally appears to be suppressed in pregnancy. Perhaps what leads to APOs is not the actions of Th17 alone, but rather, a disruption in the balance between Th17 and Treg cells.

Th2 and Tregs in Pregnancy Maintenance and Fetal Tolerance

Although Th2-type immunity dominates during normal pregnancy, loss of IL-4, IL-5, IL-9, and IL-13 in knockout mice does not prevent a normal pregnancy (97). Some redundancy between Treg and Th2 cells in suppressing detrimental Th1 immunity may explain why Th2 does not appear to be absolutely necessary for pregnancy despite being the dominant response during normal pregnancy. For example, IL-10 can be produced by both Treg and Th2 cells.

As we have seen, some level of inflammation sustained by IFN-γ is necessary for fetoplacental implantation, but too much inflammation can lead to resorption of the embryo. Treg cells may help regulate the balance of inflammation necessary for successful implantation to occur without inducing resorption. Depletion of Tregs leads to impairment of implantation in allogenic but not syngeneic mice. As Tregs are critical only when there is allogenic mis-match between parents, this suggests Tregs may be necessary for immunological tolerance of the fetus (97). Tregs accumulate in the lymph nodes draining the uterus of mice after mating. Reduced expression of FoxP3 mRNA is associated with infertility (97). Reduced Treg function and resulting chronic inflammation have been suggested to play a role in spontaneous abortion (97). Consistently with a protective role for Tregs against excess inflammation, adoptive transfer of Treg cells into BALB/c mice can inhibit lipopolysaccharide-induced fetal brain inflammation (211).

In short, Th1-type cytokines have been associated with APOs. Th17-type cytokines have been associated with a few limited APOs, but Th17-type immunity is also dampened in pregnancy. Treg and Th2 cells share some characteristics, including production of IL-10, and have both been associated with fetal tolerance and maintenance of pregnancy.

Summary of Relevant Background

The presence of periodontal pathogens in human placentas associated with APOs supports the hypothesis that periodontal pathogens can spread from the oral cavity and colonize placental tissue. This might occur before any second trimester interventions such as SRP. $CD4^+$ Th cells could therefore be primed by antigens from periodontal pathogens in the oral cavity or placenta. Recirculating memory Th cells could be reactivated by periodontal pathogens in the placenta, inducing local inflammation through cytokines such as IFN-γ.

Given that systemic IFN-γ has been associated with multiple APOs, a pro-inflammatory T cell response against periodontal pathogens in the placenta could impair fetal nutrient transfer or induce placental apoptosis. What is currently unknown is whether $CD4^+$ T cells responding to oral pathogens are responsible for the systemic increase in IFN-γ that has been associated with APOs. The possibility remains that other IFN-γ-producing cell types could be responsible for the observed increase in systemic IFN-γ.

Hypothesis

An increase in frequency of *P. gingivalis*-specific $CD4^+$ T cells producing Th1 associated cytokines (IFN-γ) is correlated with low birth weight in C57BL/6J mice.

Specific Aims

To test the hypothesis**,** we pursued two specific aims:

Specific Aim 1.

Determine the association between phenotype of *P. gingivalis*-specific $CD4^+$ T cells and fetal weight in C57BL/6J and BALB/cJ mice orally colonized with *P. gingivalis*.

The rationale for this aim is that, while systemic increases in IFN-γ have been associated with APOs, it has not been proven that IFN-γ-producing T cells responding to periodontal pathogens lead to low fetal weight. Utilizing mice that are and are not expected to have a Th1 bias in response to oral colonization with *P. gingivalis* will allow us to answer this question.

The working hypothesis is that the increasing frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ will negatively correlate with fetal weight in C57BL/6J mice. In Th2 biased BALB/c mice, the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-4 is hypothesized to either have no correlation or a positive correlation with fetal weight.

Specific Aim 2.

Determine the impact of *P. gingivalis*-specific effector Th1 and Th17 cells on pregnancy outcomes during oral colonization with *P. gingivalis*.

The rationale for this aim is to determine whether Th1-biased mice and Th17-biased mice yield different APOs on the same genetic (H-2^b) background. The working hypothesis is that frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A will be independent of fetal weight in C57BL/6NCrl (Th17-biased) mice whereas increasing IFN-γ production will correlate with low fetal weight in C57BL/6J. This was based on evidence that Th17 is involved in some APOs, but not low birth weight.

To achieve these Specific Aims, we pursued these goals:

1. Identified strains and substrains of mice that were susceptible to fetal weight reduction after oral colonization with *P. gingivalis.*
2. Detected the presence of *P. gingivalis* in the placenta to determine the potential for re-stimulation of placental T cell.
3. Determined the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 in uterine-draining PaLN and oral-draining CLN.
4. Correlated the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 to fetal weight outcomes in mice susceptible or resistant to alveolar bone loss.

Materials and Methods

Mouse Strains and Expected T Cell Phenotypes

Utilizing different substrains from different vendors, we tested whether any of the Th cell phenotypes correlated with the consequent APOs.

Specific Aim 1. C57BL/6 and BALB/c mice: Th1- versus Th2-bias

The murine strains selected in this aim are well-characterized as mouse models of periodontitis. Specifically, C57BL/6J are resistant to alveolar bone loss when orally colonized with *P. gingivalis* strain ATCC 53977 and are historically defined as Th1-biased (IFN-γ) (70, 212). BALB/cJ are susceptible to alveolar bone loss and are considered Th2-biased (IL-4) (70, 199, 200, 212-214). Using these models of alveolar bone destruction, we expected that C57BL/6J mice would be susceptible to low fetal weight because of their Th1-bias, whereas in BALB/c mice the Th2 response would lead to fetomaternal tolerance.

Specific Aim 2. C57BL/6J versus C57BL/6NCrl mice: Different Frequency of Small Intestine Lamina Propria Th17 Cells

In C57BL/6N mice that harbor intestinal segmented filamentous bacterium (SFB), 10-15% of small intestine lamina propria (SI-LP) $CD4^+$ T cells express IL-17A. SFB positive C57BL/6 mice include those from Taconic Farms (NTac) and Charles River Laboratory (NCrl) (215).

By contrast, C57BL/6 mice from Jackson Laboratory (J) lack SFB, and thus only 1-2% of their SI-LP $CD4^+$ T cells express IL-17A (215). C57BL/6J from Jackson Laboratory are therefore considered “Th17 deficient” in the SI-LP. When C57BL/6J mice from Jackson are co-housed for two weeks with mice that have SFB in their intestinal microbiota, the numbers of Th17 cells within their intestines increase in number to close to those of mice that were SFB colonized from birth (215). This suggests that the difference in Th17 cell induction is related to the microbiome and not to genetic differences between C57BL/6 vendor substrains.

It is reasonable to hypothesize that SFB regulation of Th17 cell generation in the intestine can influence Th17 cell development at distant niche as the oral cavity. For example, depletion of SFB with vancomycin leads to decreased IL-17 production in murine lungs (216), with the caveat that vancomycin is not selective for SFB alone. Introduction of SFB into the intestines of germ-free K/BxN mice leads to the development of Th17 cells associated with autoimmune arthritis (217, 218). Intestinal SFB colonization has also been demonstrated to protect against development of diabetes in female non-obese diabetic mice (219), suggesting that intestinal priming of Th17 cells in response to SFB colonization can have effects at distant sites. However, the influence of SFB colonization on Th17 cell development in the oral mucosa has not been established.

In addition to the presence or absence of SFB in the microbiota, it must be noted that mice housed as Jackson have been separated for many generations

from mice housed at Charles River. C57BL/6J and C57BL/6N (NTac or NCrl) have been described as having differences in metabolic phenotypes, weight regulation, and inflammatory immune responses (220, 221). It is difficult to determine, without using co-housed mice, the exact role that genetic drift between the two substrains versus the microbiota of each substrain, plays in the observed phenotypic differences.

SFB and Th17 in BALB/c mice

Importantly, the effect of SFB on Th17 development in BALB/c mice has not been established. Thus, both, BALB/cJ and BALB/cAnNCrl mice were evaluated for both fetal weight and Th cell phenotypes, in order to determine whether Th17 cells were increased in BALB/c mice from Charles River over BALB/c mice from Jackson, and whether a difference in Th17 might lead to a difference in fetal weight outcomes (Table 4). Vendor substrain differences in BALB/c mice have been noted in a limited number of disease models (222, 223) but BALB/c mouse substrain genetic and microbiota differences have not been as fully characterized as C57BL/6 mouse substrains.

Specific Aim 2. C57BL/6UofM mice: *P. gingivalis*-specific IL-17A and IFN-γ Production by $CD4^+$ T Cells in the Draining Cervical Lymph Nodes

Finally, our lab raises C57BL/6 mice originally purchased from Jackson Laboratories several generations ago. Therefore, their microbiota and genetics

cannot be assumed to be identical to original Jackson substrain. These mice have been used as wild-type littermate controls for transgenic mice and are designated C57BL/6UofM (Table 4).

We determined that C57BL/6UofM mice have *P. gingivalis* gingipain-specific Th17 cells their cervical draining lymph nodes (CLN) 28 days after initial oral colonization with *P. gingivalis* (224). The frequency of *P. gingivalis*-specific Th1 cells gradually increases after day 28 of persistent *P. gingivalis* colonization, while the frequency of Th17 cells remains relatively heightened and constant over time. C57BL/6UofM were therefore utilized to correlate concurrent production of IL-17A and IFN-γ in $CD4^+$ T cells with fetal weight outcomes.

Table 4. Expected T cell Substrain Phenotypes and SFB Colonization

Substrain	*P. gingivalis*-specific T cell response (predicted)	SFB colonization
C57BL/6J	Th1 – IFN-γ	No
C57BL/6NCrl	Th17 – IL-17A	Yes
C57BL/6UofM	Th17/Th1– IL-17A/ IFN-γ	To Be Determined
BALB/cJ	Th2 – IL-4	No
BALB/cAnNCrl	Th2 – IL-4	Yes

Animal Husbandry

C57BL/6UfoM mice were bred in-house. All other mice were purchased from their respective vendors. Mice were housed in microisolator cages in accordance with University of Minnesota and National Institutes of Health guidelines. All experiments were performed on female (8–10 weeks) mice with vendor substrain-matched male wild type mice used for mating. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota Protocol # 0912A75416, 1211A24161, 151133178A.

P. gingivalis Culture and Inoculation

P. gingivalis strain ATCC 53977 was grown on blood agar plates as previously described (212, 224). *P. gingivalis* was harvested and re-suspended in 2% low viscosity carboxymethylcellulose (Sigma) such that each mouse received 4 x 10^9 CFU per 50 µl inoculation by oral gavage. Two percent carboxymethylcellulose alone was used as sham vehicle control. Inoculation began after 10 days of sulfamethoxazole and trimethoprim (SMZ) pre-treatment followed by four days of water only as previously described (212). Mice were inoculated every 4 days until termination of experiment. The timeline of inoculation and mating is included as Figure 1.

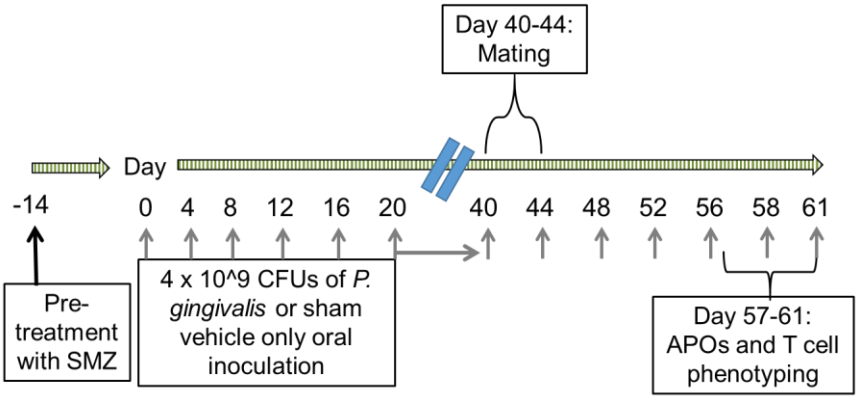


Figure 1. Experimental Timeline. Time course of oral inoculations with *P. gingivalis*, mating, and harvesting of pregnant and non-pregnant female tissues and fetuses.

Mating and Evaluation of Copulation Plug

Forty days after initial oral inoculation, female mice were cohoused with vendor substrain and age matched naïve male mice at a ratio of one male to two females. Copulation plugs were evaluated each morning for four days, representing the duration of one mouse estrus cycle, after addition of females to male cage. Females were removed from males at the first instance of an observable population plug or after four days/one estrus cycle without observable plug, whichever came first. Day zero of pregnancy was determined by the presence of a copulation plug and subsequent separation from the male.

Isolation of Lymph Nodes and Maternal Tissue

On Day E17 of gestation, or equivalent in females that plugged but did not become pregnant, mice were euthanized by CO_2 inhalation. The uterus, with or without accompanying fetuses, was immediately removed. Maternal draining cervical lymph nodes (CLN) and para-aortic (or lumbar, PaLN) lymph nodes were collected for ELISpot. Maternal blood, liver, and spleens were also harvested for later PCR analysis. All tools used for placenta dissection were initially cleaned with 10% bleach followed by DNA-Out (G-Biosciences) between procedural steps.

ELISpot

Maternal PaLN-CLN were reduced to single cell suspensions as previously described (224). $CD4^+$ T cells were purified using a commercially available kit per company instructions, with LS type purification columns (Miltenyi-Biotech 130-104-454, 130-042-401). Recovered cells were routinely a minimum of 94% $CD4^+$ T cells. $CD4^+$ T cells were counted and plated at 3×10^5 $CD4^+$ T cells/well in a 96 well plate (EMD Milipore MAIPS4510) pre-coated with IL-17A, IFN-γ, or IL-4 ELISpot capture antibodies (Ebio17CK15A5, AN-18, 11B11, Thermo Fisher). 3×10^5 irradiated antigen presenting cells (2000 rads, splenocytes) from a naïve strain-matched mouse were subsequently added to the purified $CD4^+$ T cells and mixed in Click's Medium supplemented with heat-inactivated FBS (Atlas Biological) L-glutamine, 2-mercaptoethanol, and antibiotics as previously described (224).

To the negative control wells, media only was added. 50ng/ml phorbol myristate acetate (PMA)/ plus 500ng/ml Ionomycin (Sigma) was added to positive control wells. 1×10^8 CFU of freeze-killed *P. gingivalis* was added to all other wells in order to determine the memory $CD4^+$ T cell response to *P. gingivalis*. After 40 hours of incubation at 37 degrees C and 5% CO2, cells were washed away, leaving only cytokines bound to the plate membrane surface. Biotin-conjugated anti IL-17A, IFN-γ, or IL-4 detection antibodies (Ebio17B7, R4-6A2, BCD6-2462, Thermo Fisher) were incubated on the plates for 3 hours at room temperature. Detection antibodies represented different clones against

different epitopes, with different binding sites on the cytokine from capture antibodies.

Spots were revealed with avidin-horseradish peroxidase (Thermo Fisher) followed by 3-amino-9-ethylcarbazole (AEC) substrate for ELIspot (BD Biosciences part #551951). Each spot was determined to represent one T cell producing a cytokine of interest, or one spot forming unit (Figure 2). Spot counts were determined using Cellular Technology Limited (CTL) branded Immunocapture and ImmunoSpot® software with SmartCount™, hair (or debris) removal, 95% of well area analyzed and subsequently processed for quality control. Edges of wells were excluded to avoid picking up spots caused by edge effects.

We attempted to evaluate IL-10 production via ELISpot as well, however, there was a very high non-antigen specific background production of IL-10 in both sham and *P. gingivalis* colonized mice, even without any *in vitro* re-stimulation. This was true for both pregnant and non-pregnant females.

The subset of females used for ELISpot were determined to have fetal weights representative of total experimental dams. On a final note, some females that were pregnant without a known copulation plug date were used to increase ELISpot numbers comparing pregnant sham- and *P. gingivalis*-colonized females but could not be used to correlate ELISpot cytokine results to fetal weight as the exact gestational ages of their fetus were unknown.

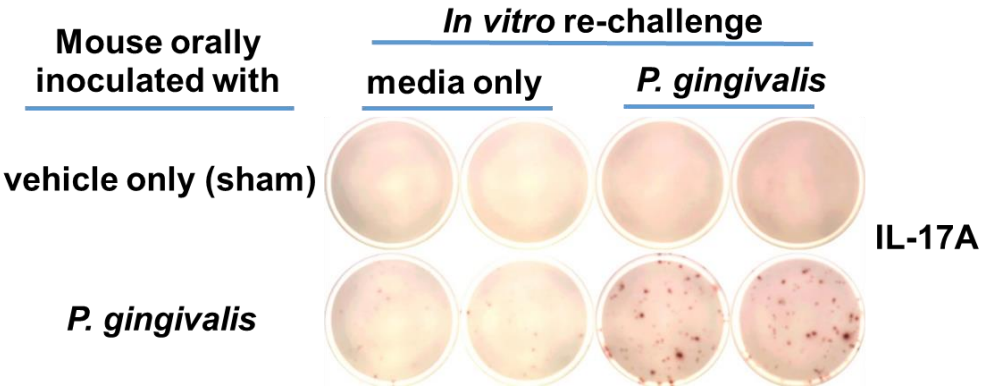


Figure 2. Example of ELISpot Plate Readout for IL-17A. 3 x 10^5 purified $CD4^+$ T cells from sham or *P. gingivalis* orally colonized mice were plated wells pre-coated with functional grade purified anti-IL17A. 3 x 10^5 irradiated splenocytes from strain-matched naïve mice were added and cells were thoroughly mixed by gentle pipetting followed by gentle rocking. Finally, media only, freeze-killed *P. gingivalis*, or PMA/Ionomycin positive control (not shown) were added to wells in duplicate. After 40 hours incubation, cells were washed off and cytokine production was revealed with anti-IL17A biotin, avidin-horseradish peroxidase, and AEC substrate. Plates were imaged and counted on a CTL ELISpot classic imager, with proprietary CTL counting software.

Isolation of Fetus and Placenta

All tools used for placenta dissection were initially cleaned with 10% bleach followed by DNA-Out (G-Biosciences) between procedural steps. The uterus and fetuses were removed. The uterine chain was washed three times in three changes of sterile PBS to remove external maternal bacterial and blood contamination. Each fetus and placenta was separated into individual wells of a 24-well tissue culture dish (Corning). An individual 18 gauge needle was used to separate the fetus from placenta. Fetuses were patted dry to remove excess amniotic fluid, weighed, photographed, and were ultimately decapitated to ensure humane euthanasia (IACUC protocol). Each pair of placentas of one litter were divided in half. The first half of each placenta was used for DNA purification and qPCR. The other half was either fixed in neutral buffered 10% formalin (Cancer Diagnostics) for immunohistology or frozen in RNAlater (Sigma-Aldrich) for archival purposes and future RNAseq.

DNA Purification

DNA was purified from placenta samples using a MasterPure DNA Purification Kit (Epicentre) per manufacturer protocol with modifications. Samples were placed in 300 µL of Tissue Lysis Buffer containing 50 µg of proteinase K and allowed to incubate overnight at 55 °C in a water bath. One hundred seventy-five µL of protein precipitation solution was added after samples were cooled to room temperature. After centrifugation, the supernatant was

removed and cleaned with washes of isopropanol and ice cold 70% ethanol. DNA pellets were re-suspended in 500 µL of TE buffer (Genemate). Concentration of DNA in samples was determined with a NanoDrop 2000 (Thermo Fisher). Samples were uniformly re-suspended at 50 ng/µL of total DNA. Laboratory tools and disposables used for DNA techniques and qPCR were kept separate from tools and disposables used to prepare bacteria or murine tissue harvesting. Barrier tips were also exclusively used to prevent DNA carry over on pipet surfaces. Pipets and other equipment were treated with 10% bleach and DNA Out to avoid carry over of DNA from one reaction to another.

Pure cultures of *P. gingivalis* were processed in the same manner for positive controls. Ear and tail cuttings from naïve, unexposed weanlings and breeding males were processed by the same technique to serve as negative and contamination controls. Standards were prepared by isolating genomic DNA from sham inoculated or naïve mouse ears or tails and spiking them with known amounts of DNA from pure cultures of *P. gingivalis* strain 53977.

Conventional Nested PCR for Presence of *Pg* in Placentas

A: External nested PCR primers. GoTaq Clear Master Mix (Promega) was combined with 0.5 µM of each external primer (Table 5) and 250 ng of total DNA. The first round of amplification was performed on a Mastercycler Gradient thermocycler (Eppendorf) with the following conditions: 95 °C for 2 minutes,

followed by 26 cycles of 95 °C for 30 seconds, 54 °C for 2 minutes, and 72 °C for 3 minutes.

B: Internal nested PCR primers: Reaction produced by amplification “A” (just above) was diluted 1/200. GoTaq Green Master Mix (Promega) was combined with 0.5 µM of each internal primer (Table 5) and 5 µl of 1/200 dilution from step A. Second round amplification was performed on a Mastercycler Gradient (Eppendorf) with the following conditions: 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 3 minutes.

Products from reaction B were run on a 2% agarose gel to identify *P. gingivalis*-specific DNA amplicons.

Table 5. External and Internal Nested Primers

Reaction A & B. Primer name	Nucleotide sequence	Reference
A. External F 5’-3’	GTGAGGTAACGGCTCACCAA	Created with NCBI primer blast
A. External R 5’-3’	AATATGGCTTTTCGCCGTGC	Created with NCBI primer blast
B. Internal F 5’-3’	AGGCAGCTTGCCATACTGCG	(152, 225-228)
B. Internal R 5’-3’	ACTGTTAGCAACTACCGATGT	(152, 225-228)

Conventional PCR as Template for Nested qPCR.

The first round of amplification for nested qPCR was identical to the first round of amplification for nested conventional PCR. GoTaq Clear Master Mix (Promega) was combined with 0.5 µM of each external primer (Table 5, “A” primers) and 250 ng of total DNA. Samples with known volumes of mouse genomic DNA plus known volumes of *P. gingivalis* DNA were used to establish a standard curve for downstream qPCR analysis. The first round of amplification was performed on a Mastercycler Gradient thermocycler (Eppendorf) with the following conditions: 95 °C for 2 minutes, followed by 26 cycles of 95 °C for 30 seconds, 5 °C for 2 minutes, and 72 °C for 3 minutes. Pipets and other equipment were treated with 10% bleach and DNA Out to avoid carry over of DNA from one reaction to another.

Quantitative PCR (qPCR) with Nested Internal Primers

First round samples were diluted 1/200 in nuclease free water after amplification. Maxima SYBR Green/ROX qPCR (Thermo Fisher) master mix was combined with 0.5 µM of each internal primer and 5 µl of 1/200 diluted sample from conventional PCR with external primers (Table 5, “B” primers). Each sample was prepared in duplicate. Samples were processed on an Mx3000p qPCR thermocycler (Agilent Technologies) with the following conditions: Normal 2-step qPCR was selected with an annealing temperature of 60 °C x 1 minute for 36 cycles, followed by standard melt curve analysis. All

other parameters were as recommended by qPCR master mix instructions. Nested qPCR was determined to be approximately 10-fold more sensitive than conventional nested PCR. Nested qPCR could detect *P. gingivalis* in samples that were deemed negative by conventional, non-nested qPCR. Increasing amounts of starting total DNA decreased sensitivity.

Pure *P. gingivalis* DNA produced a melt curve peak around 84 °C (Figure 3). Ng of *P. gingivalis* DNA detected were converted into equivalent CFU of *P. gingivalis* present necessary to produce that much *P. gingivalis* DNA (Figure 4).

Statistical Analysis of Nested qPCR.

Threshold cycle (Ct) is the cycle number at which the fluorescence generated within a reaction crosses the threshold. Ct is inversely correlated to log value of initial copy number of the template DNA. DRn (Δ Rn) is the magnitude of the SyBR green fluorescence signal generated during the qPCR at a given time point, when normalized to a reference value generated by the reference dye ROX for this data set. Fluorescence -R'(T) is a value generated by DNA-binding dye SyBR green in the qPCR assay. As the amplicon fragment is heated a sudden decrease in fluorescence is detected when the melting temperature (Tm) is reached. The temperature at which SyBR green loses fluorescence is displayed as a peak on the melt curve analysis. A non-linear regression fit for line x is log and y is linear was used to calculate the standard

curve, where x was ng of *P. gingivalis* DNA and y was threshold cycle (Ct). The slope of the standard curve is used for efficiency calculation. A slope of -3.32 indicates ideal efficiency of the qPCR reaction (within the range of -3.1 to -3.6 is generally considered acceptable).

Statistical analysis of standard curves and unknowns was done with Prism 6.0 (GraphPad). Prism was also used to interpolate unknown values from the standard curve. Mass of *P. gingivalis* DNA in ng per 250 ng of total DNA was correlated with fetal weight using Spearman's correlation.

Analysis of SFB in Intestinal Samples

To isolate DNA, samples of intestine and placentas were digested as described above. Isolated total DNA (genomic mouse plus bacterial) was analyzed via PCR as described in (229).

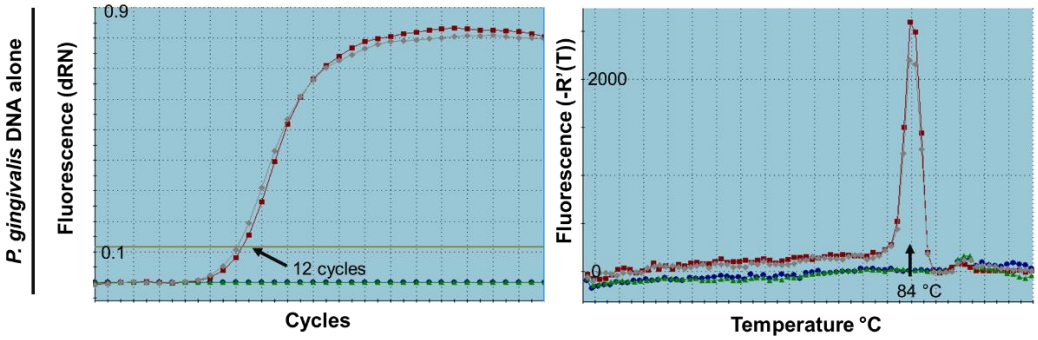


Figure 3. Nested qPCR. A) Amplification curve which displays the cumulative magnitude of fluorescence signal of DS DNA at the end of each cycle. The cycle at which the threshold is crossed (*i.e.* 12 cycles arrow) from two independent samples is dependent on the amount of target DNA sequence being amplified. B) Temperature at which a significant decrease in SyBR green fluorescence is detected displayed as an inverted peak. *P. gingivalis* DNA after nested qPCR produced a melt curve peak around 84 °C using the given primers.

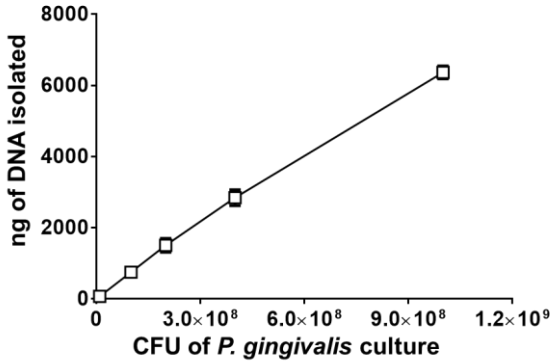


Figure 4. Recoverable DNA from Serial Dilutions of Pure Cultured *P. gingivalis*.

OD600 was used to determine the colony forming units (CFU) of *P. gingivalis* in suspension based on growth curves from serial dilutions. DNA was extracted using the same techniques and reagents as the mixed tissue-bacterial samples. Total ng of DNA that could be recovered across multiple serial dilutions was determined. Three independent replicates of this experiment were performed. An average of these values was used to convert ng of DNA identified by qPCR into equivalent CFU of *P. gingivalis* present.

Results

P. gingivalis is Detected in Murine Placentas after Mucosal Colonization

Given the failure of oral-only focused treatment to reduce the risk of APOs, we deduced that the association between periodontitis-associated pathogens and APOs must be assessed in the reproductive organs. We tested the hypothesis that periodontal pathogens such as *P. gingivalis* were present in the placenta or uterine tissues in association with low fetal weight. The hypothesized mechanisms by which *P. gingivalis* in the placenta could lead to APOs were either direct damage of the fetoplacental unit via virulence factors or initiation of an inflammatory immune response against *P. gingivalis* that lead to indirect impairment of placental nutrient transfer. To test whether *P. gingivalis* can be detected in the placental tissue, conventional nested PCR was used to evaluate the presence or absence of *P. gingivalis* DNA in murine placental tissue after oral colonization of the dam with *P. gingivalis*.

P. gingivalis DNA was detected in most placentas from orally colonized C57BL/6J dams (Figure 5). *P. gingivalis* DNA was not detected in placentas of sham colonized dams (Figure 5). In dams that were orally colonized with *P. gingivalis* there was a significant reduction in fetal weight ($p \leq 0.05$) as compared to dams that were sham colonized (Figure 5).

Within the dams that were orally colonized with *P. gingivalis* ultimately average fetal weight in placentas where *P. gingivalis* was detected and placentas where *P. gingivalis* not detected by conventional nested PCR were similar (Figure 5). *P. gingivalis* DNA could not be detected in uterine tissues from these mice, even when *P. gingivalis* DNA was detected in placentas from the same dam (data not shown).

Given that mere presence of *P. gingivalis* DNA in the placenta did not explain fetal weight outcomes, we next questioned whether the *amount* of *P. gingivalis* DNA in the placenta, and not just presence, might be correlated with fetal weight outcomes.

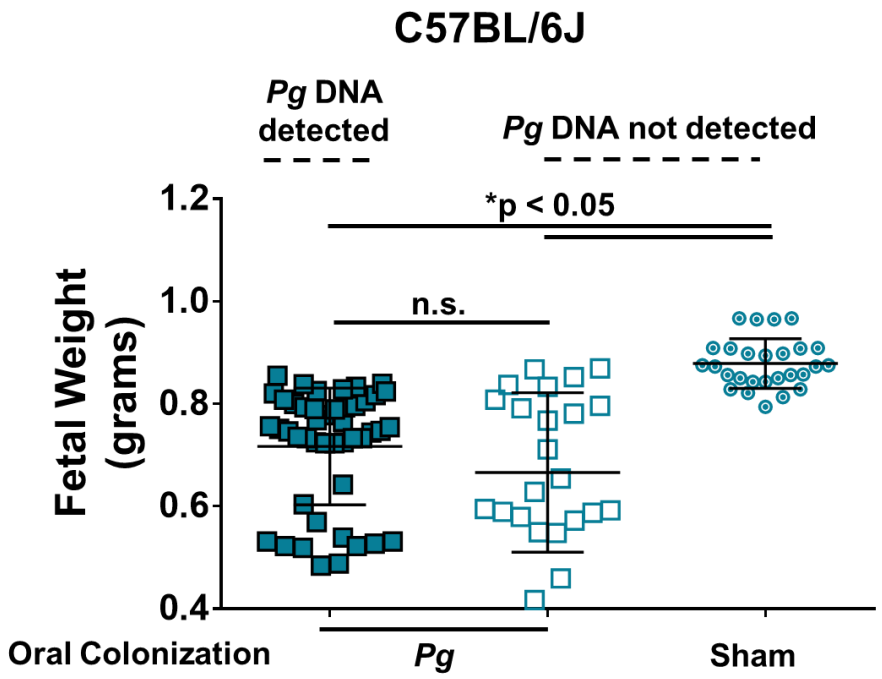


Figure 5. *P. gingivalis* DNA can be Detected in the Placentas of C57BL/6J Dams after Oral Colonization with *P. gingivalis*. Sham- or *P. gingivalis*-colonized C57BL/6J females were harvested on day E17 of gestation from the date the copulation plug was observed. Placentas were harvested and total DNA was isolated for conventional nested PCR with primers specific for a region spanning between the 16s and 23s rRNA genes of *P. gingivalis*. Fetal weight was determined after removal of amniotic fluid, sac, and placenta. 24 tested placentas from *P. gingivalis* orally colonized mice were negative for *P. gingivalis* DNA; 46 were positive. Data is shown as average fetal weight ± SD. * = Student's *t* test $p \leq 0.05$

Increasing Amounts of *P. gingivalis* DNA in C57BL/6J and C57BL/6UofM Placentas Correlates with Decreasing Fetal Weight

The results for presence of *P. gingivalis* DNA in the placenta raised two possibilities. First, some low fetal weight placentas may have had *P. gingivalis* DNA present, but there was not enough to meet the limit of detection of the conventional nested PCR assay. The other was that presence of *P. gingivalis* in the placenta, as proxy measured by amount of *P. gingivalis* DNA, might be less important than the amount of *P. gingivalis* present. Indeed, this is the situation in the oral cavity, where *P. gingivalis* is normally found at low levels in the orally healthy individual and significantly higher levels in the periodontally diseased individual. We thus decided to use a more sensitive nested quantitative PCR (nested qPCR) methodology to assess the amount of *P. gingivalis* DNA present in murine placentas. This would allow us to potentially detect bacteria in placentas that were negative by conventional nested PCR and quantitate an estimate of the bacterial burden per placenta. Assay quality control was performed by spiking naïve mouse genomic DNA with known amounts of *P. gingivalis* genomic DNA (Figure 6).

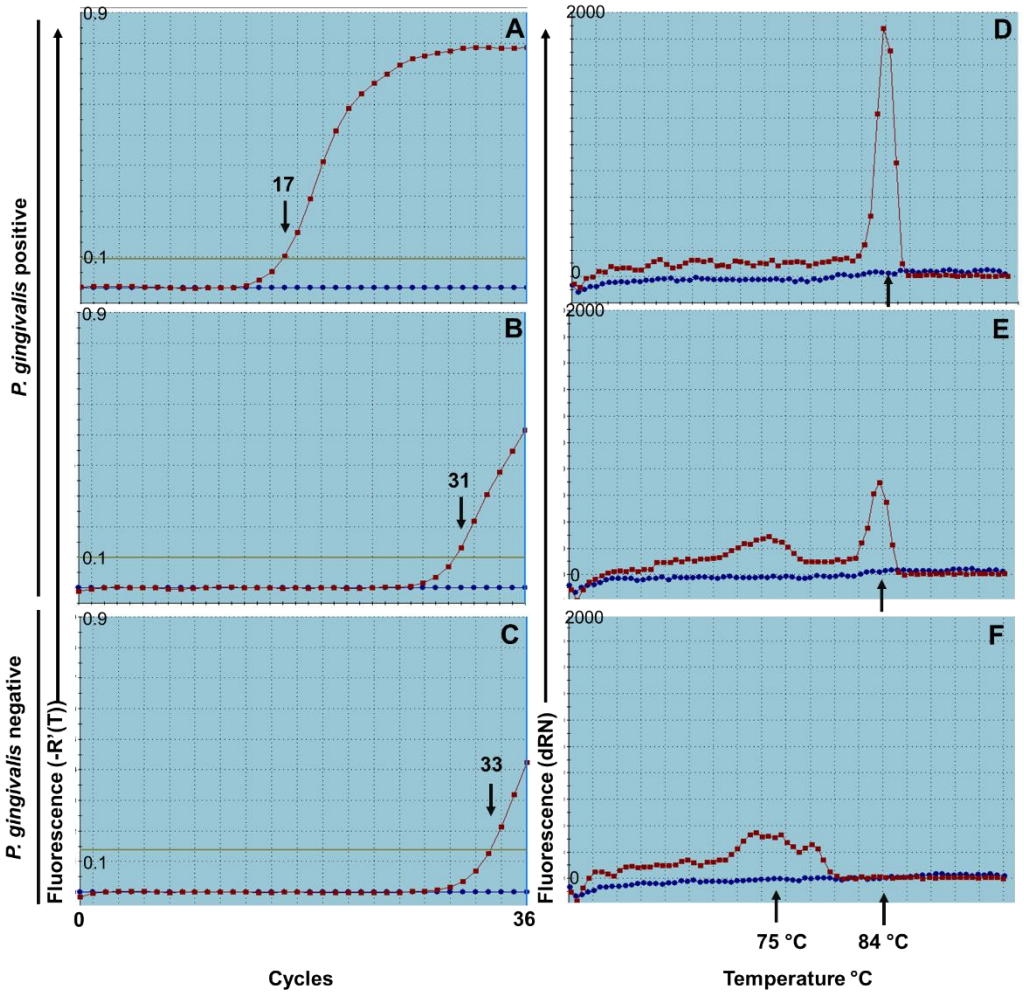


Figure 6. Nested qPCR for *P. gingivalis* DNA in Placentas of C57BL/6J Mice.

Detection of a *P. gingivalis* specific sequence within 250 ng naïve mouse genomic DNA: 7 ng (1 x 10^6 equivalent CFU) (A), 0.7 pg (equivalent 100 CFU) (B) or no *P. gingivalis* DNA (C). 7 ng of *P. gingivalis* DNA present in mouse genomic DNA produced a narrow melt curve that peaks at 84°C only (D). Lower amounts of *P. gingivalis* DNA (0.7 pg) yielded two melt curves, a wide peak at 75°C and a narrow *P. gingivalis*-specific peak at 84°C (E). When *P. gingivalis* DNA is absent (F) only a wide 75°C melt curve was produced due to excess of primers and potential mispriming.

Nested qPCR had a lower limit of detection than conventional qPCR revealing that some samples that were *P. gingivalis* negative with conventional nested PCR were positive with nested qPCR. Two-thirds of C57BL/6J mouse placentas were ultimately found to contain *P. gingivalis* DNA by nested qPCR, with a limit of detection equivalent to 10 CFU of *P. gingivalis* per 250 ng of placental DNA. Increasing amounts of *P. gingivalis* DNA in C57BL/6UofM and C57BL/6J placentas were correlated with decreasing fetal weight (Figure 7A and 7B). *P. gingivalis* DNA was not detected in any placentas from sham-inoculated females (data not shown).

We can therefore conclude that *P. gingivalis* in the placenta represents a hypothetical antigen pool that would be effective in attracting and re-stimulating *P. gingivalis*-specific memory $CD4^+$ T. This could result in the release of cytokines such as IFN-γ, which has been shown to be detrimental to pregnancy outcomes, into the placental tissue.

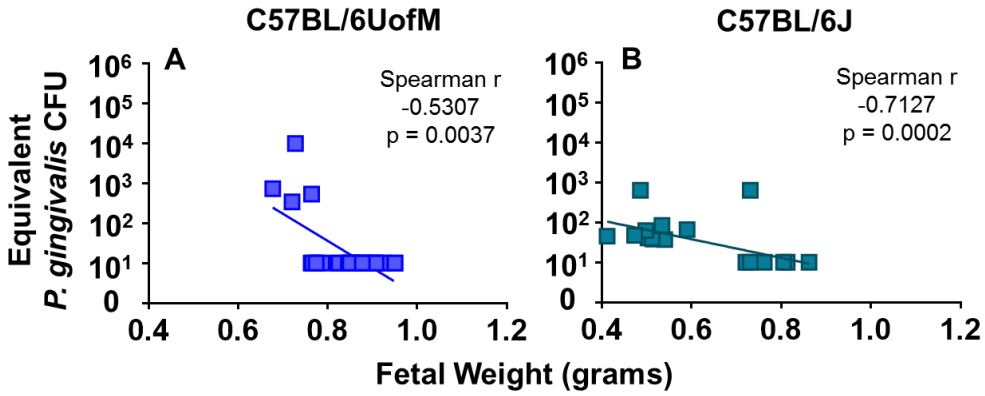


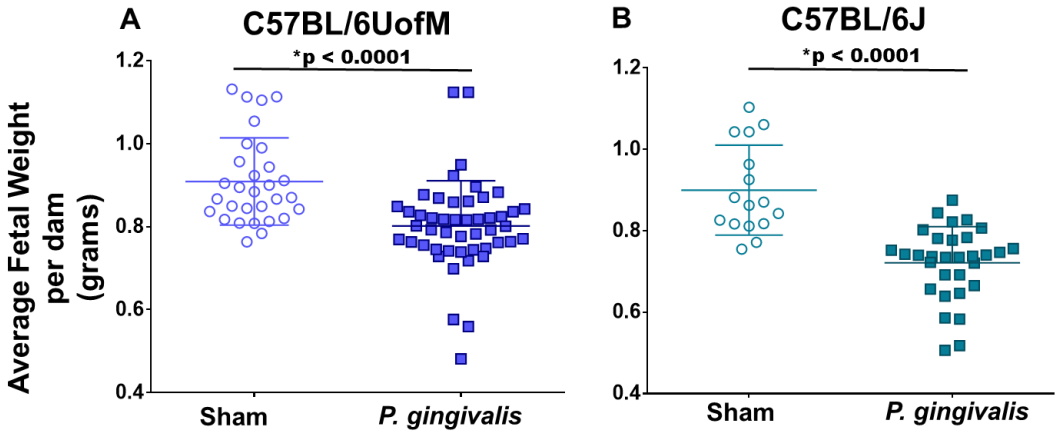
Figure 7. Increasing Amounts of *P. gingivalis* DNA in Placentas is Correlated with Reduced Fetal Weight. Nested PCR of placental tissue from C57BL/6UofM (A) and C57BL/6J (B) using primers specific for the intergeneric region between the 16s and 23s rRNA genes of *P. gingivalis* were utilized to correlate fetal weight and amount of *P. gingivalis* DNA. Nanograms of *P. gingivalis* DNA per 250ng of placental DNA in this assay was determined using a standard curve generated from naïve mouse DNA spiked with known amounts of *P. gingivalis* pure culture DNA. All samples for which no *P. gingivalis* DNA could be detected were set as the limit of detection (0.07 pg or 10 CFU of *P. gingivalis* DNA per 250 ng total placental DNA). Spearman correlation was used to compare amounts of *P. gingivalis* DNA (measured in ng) with fetal weight (grams). Each data point represents the weight one fetus (*x*) to *P. gingivalis* DNA in the corresponding placenta (*y*). Data was converted to equivalent CFU of *P. gingivalis* to demonstrate clinical relevance.

C57BL/6Uof M and C57BL/6J Mice Experience Low Fetal Weight in Response to Oral Colonization with *P. gingivalis*

C57BL/6JUofM mice were derived from C57BL/6J mice several generations ago, meaning that while they are similar to one another, the two substrains cannot be assumed to perfectly identical either in genetics or microbiome. Both show a correlation between increasing *P. gingivalis* DNA in placentas and decreasing fetal weight.

C57BL/6J mice have been characterized as unable to develop Th17 in the lamina propria of the small intestine (Th17-deficient). This deficiency is credited to the absence of SFB microorganisms in the ileum. Virgin and male C57BL/6UofM mice have a dual IL-17A/IFN-γ response to *P. gingivalis* in their CLN after chronic *P. gingivalis* colonization (greater than 35 days) with repeated inoculations (224).

P. gingivalis colonized C57BL/6UofM and C57BL/6J females experienced significantly lower average fetal weight per dam than sham colonized females (Figure 8). Although significant for both substrains, fetal weight reduction was more severe in C57BL/6J than in C57BL/6UofM dams after oral colonization with *P. gingivalis*.



Parameter	C57BL/6UofM Sham vs *P. gingivalis*	C57BL/6J Sham vs *P. gingivalis*
Fetal Weight	0.909 vs 0.802 g *p < 0.0001	0.900 vs 0.722 g *p < 0.0001
n Sham dams	30	16
n P. gingivalis dams	37	31

Figure 8. Reduced Fetal Weight in C57BL/6UofM and C57BL/6J mice in Response to Oral Colonization with *P. gingivalis*. Fetuses were harvested on day E17 from dams that were orally colonized with *P. gingivalis* or sham only. After removal of the placenta, amniotic sac, and amniotic fluid the fetuses were weighed. Student's unpaired *t*-test with Welch's correction was used to compare the average fetal weight per dam and average fetal weight per group. Symbols (circles and squares) show average fetal weight per dam/litter. Experimental groups shown display average ± SD.

Frequency of *P. gingivalis*-specific CD4$^+$ T cells Producing IL-17A and IFN-γ were Similar between Sham and *P. gingivalis* colonized C57BL/6UofM or C57BL/6J dams

Since C57BL/6UfoM and C57BL/6J mice experienced reduced fetal weight in response to oral *P. gingivalis* colonization (Figure 8), we hypothesized that an increased frequency of IFN-γ producing *P. gingivalis*-specific CD4$^+$ T cells in the maternal PaLN-CLN would correlate with reduced fetal weight. We utilized ELISpot assay to measure three subset-specific cytokines: IL-17A, IFN-γ, and IL-4. The frequency of *P. gingivalis* specific CD4$^+$ Th cells of a specific phenotype as measured by number of spot forming units (SFU) were compared in mice that were orally colonized with *P. gingivalis* and sham-colonized mice after *in vitro P. gingivalis* re-stimulation.

We discovered that pregnant C57BL/6UofM mice have a greater frequency of IL-4-producing *P. gingivalis*-specific CD4$^+$ T cells in PaLN-CLN after oral colonization with *P. gingivalis* (Figure 9A). Pregnant C57BL/6J mice had an equal frequency of IL-4 producing P*. gingivalis*-specific CD4$^+$ T cells after oral colonization with sham or *P. gingivalis* (Figure 9B). Sham and *P. gingivalis* orally colonized C57BL/6UofM and C57BL/6J dams had the same frequency of *P. gingivalis*-specific CD4$^+$ T cells producing IL-17A or IFN-γ (Figure 9, A and B).

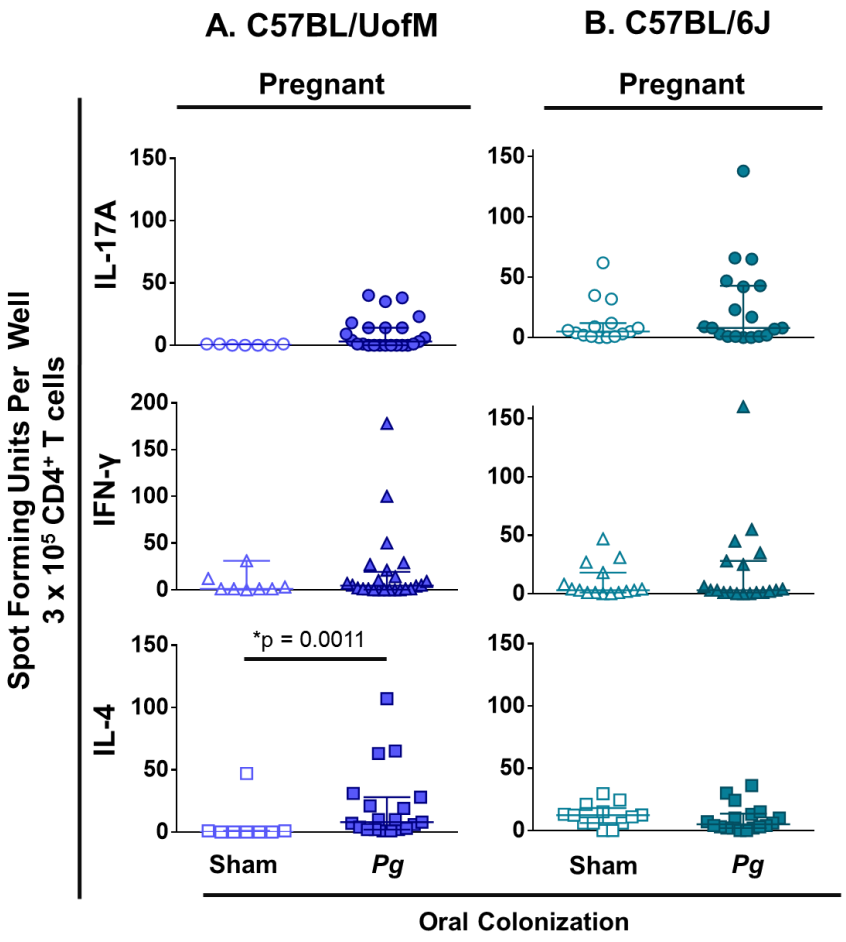


Figure 9. Frequency of $CD4^+$ T cells Producing IL-4 was Significantly Increased in Response to *P. gingivalis* in the PaLN-CLN of Pregnant C57BL/6UofM females. $CD4^+$ T cells were purified from PaLN-CLN on day E17 from dams that were sham- or orally-colonized with *P. gingivalis.* $CD4^+$ T cells were plated with irradiated naïve splenocytes for ELISpot to determine the frequency of T cells releasing IL-17A, IFN-γ or IL-4 after re-stimulation with freeze/thaw killed *P. gingivalis.* Groups were compared with Mann Whitney non-parametric test, as the cytokine data did not have a normal distribution. Median ± interquartile range is displayed.

The average fetal weight of each individual litter was then correlated with the production of cytokines in $CD4^+$ T cells in PaLN-CLN of each individual dam. The frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 in either substrain was determined to be independent of fetal weight (Figure 10, Figure 11).

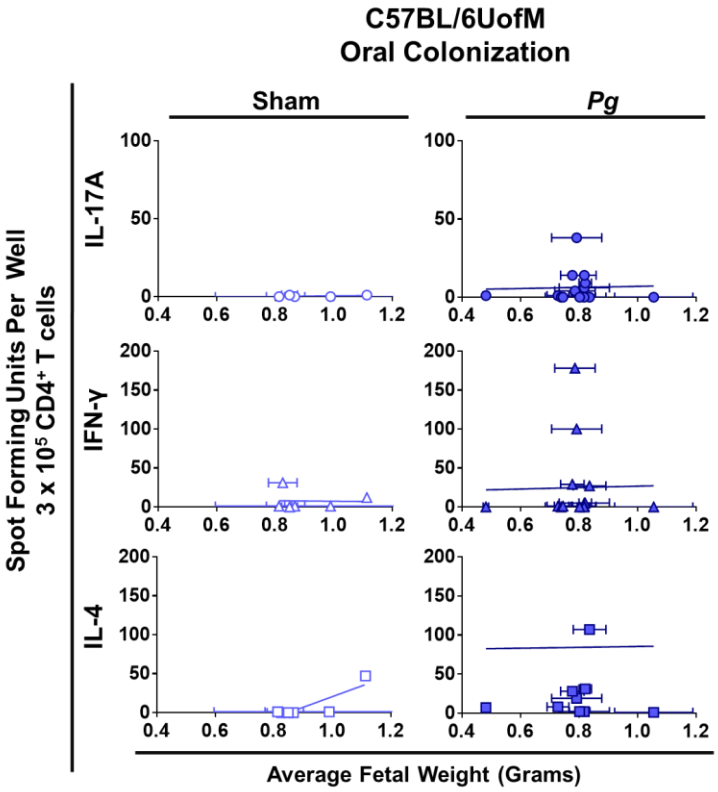


Figure 10. Fetal weight was Independent of Frequency of $CD4^+$ T cells Producing IL-17A, IFN-γ, or IL-4 in Response to *P. gingivalis* in C57BL/6UofM Dams. PaLN-CLN were harvested on day E17 from C57BL/6UofM dams that were sham- or orally-colonized with *P. gingivalis*. $CD4^+$ T cells were purified and plated with irradiated naïve splenocytes to determine phenotype of cytokine output. After removal of the placenta, amniotic sac, and amniotic fluid fetuses were individually weighed. Spearman correlation test was utilized to assess the relationship between the average fetal weight ± SD and the frequency of $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 in response to *P. gingivalis*. PMA/Ionomycin positive controls showed the plated $CD4^+$ T cells were capable of cytokine production in culture (data not shown).

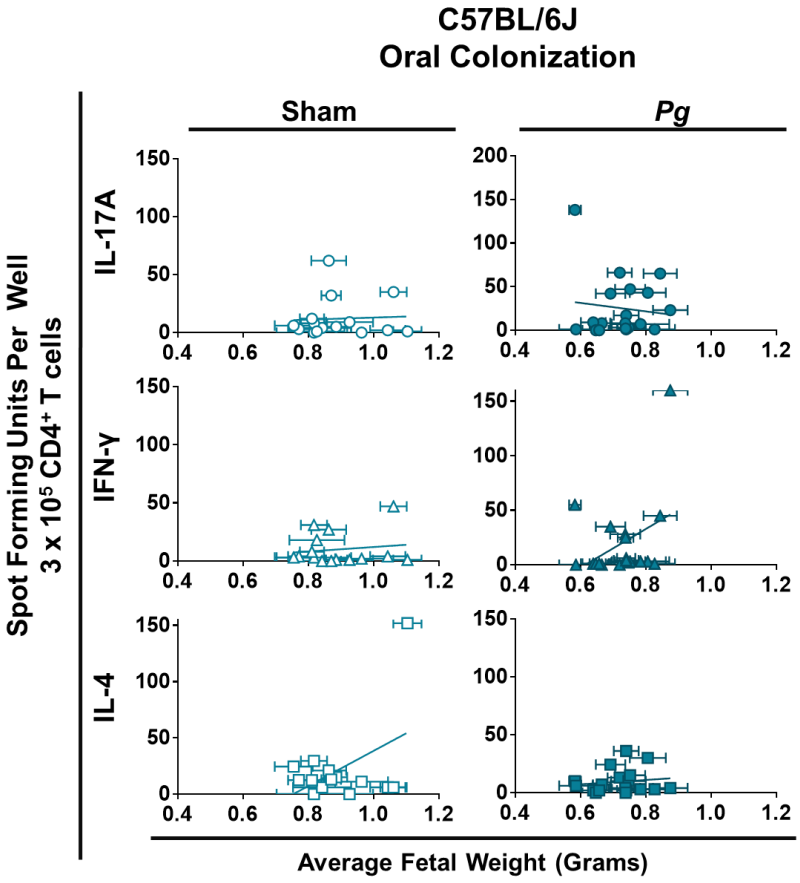


Figure 11. Fetal Weight was Independent of Frequency of $CD4^+$ T cells Producing IL-17A, IFN-γ, or IL-4 in Response to *P. gingivalis* in C57BL/6J Dams.

PaLN-CLN were harvested on day E17 from C57BL/6J dams that were sham- or orally-colonized with *P. gingivalis*. $CD4^+$ T cells were purified and plated with irradiated naïve splenocytes to determine phenotype of cytokine output. After removal of the placenta, amniotic sac, and amniotic fluid fetuses were individually weighed. Spearman correlation test was utilized to assess the relationship between the average fetal weight ± SD and the frequency of $CD4^+$ Th cells producing IL-17A, IFN-γ, or IL-4 in response to *P. gingivalis*. PMA/Ionomycin positive controls showed the plated $CD4^+$ T cells were capable of cytokine production in culture (data not shown).

Non-Pregnant C57BL/6UofM mice have Th17 and IFN-γ Producing *P. gingivalis*-specific CD4$^+$ T cells

Using a *P. gingivalis*-specific MHC class II tetramer we had previously shown that virgin female and male C57BL/6UofM mice orally colonized with *P. gingivalis* had *P. gingivalis*-specific CD4$^+$ T cells in their CLN producing IL-17A at day 28 after initial *P. gingivalis* inoculation, and both IL-17A and IFN-γ cytokines after 35 days following initial *P. gingivalis* inoculation (224). It was a mystery when the ELISpot analysis did not show a similar result in pregnant females.

We therefore decided to evaluate the effect of pregnancy on the CD4$^+$ T cell response to *P. gingivalis* after oral colonization and compared pregnant and non-pregnant females. The pregnant and non-pregnant females were compared at the same age and at the same experimental time point after oral *P. gingivalis* colonization.

Non-pregnant C57BL/6UofM females had an increased frequency of *P. gingivalis*-specific CD4$^+$ T cells producing IL-17A and IFN-γ after *P. gingivalis* oral colonization (Figure 12, A). This ELISpot results validates our previous tetramer findings in non-pregnant females. Interestingly, the parent strain C57BL/6J also had increased frequency of IL-17A-producing *P. gingivalis*-specific CD4$^+$ T cells. However, the frequency of IFN-γ producing *P. gingivalis*-specific CD4$^+$ T cells was the same whether the C57BL/6J mouse was orally colonized with *P. gingivalis* or sham (Figure 12, B).

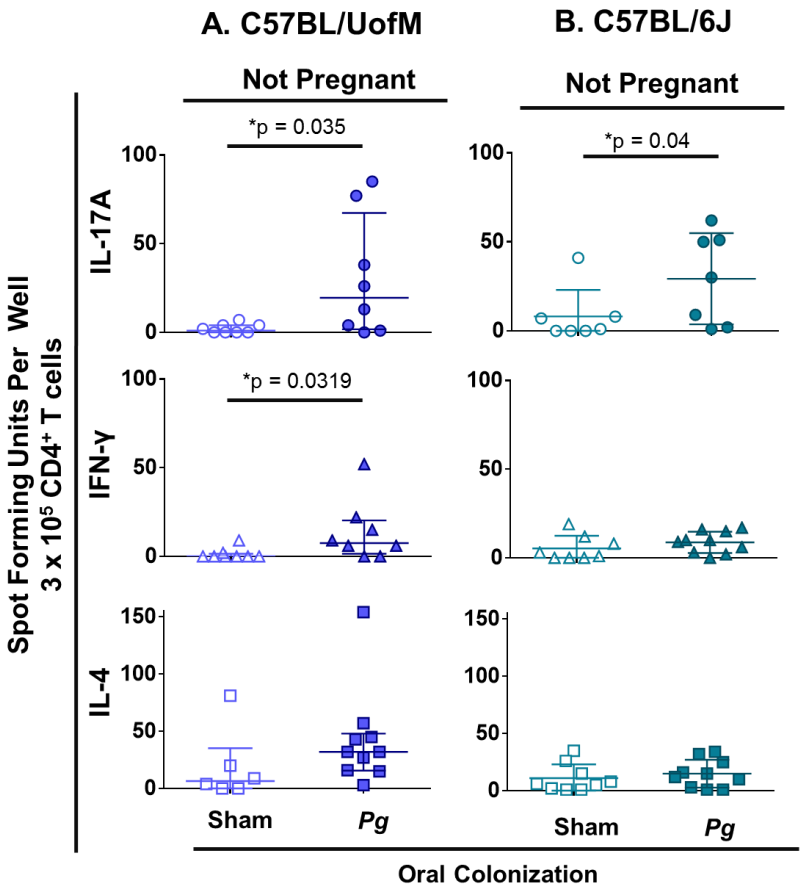


Figure 12. Frequency of $CD4^+$ T cells Producing IL-17A and IFN-γ were Significantly Increased in Response to *P. gingivalis* in the PaLN-CLN of Non-pregnant C57BL/6UofM Females. $CD4^+$ T cells were purified from PaLN-CLN on predicted day E17 from dams that were sham- or orally-colonized with *P. gingivalis.* T cells were plated with irradiated naïve splenocytes for ELISpot to determine the frequency of T cell releasing IL-17A, IFN-γ or IL-4 after re-stimulation with freeze/thaw killed *P. gingivalis.* Groups were compared with Mann Whitney non-parametric test, as the cytokine data did not have a normal distribution. Median ± interquartile range is displayed.

Summary of C57BL6/UofM and C57BL/6J Outcomes

Both C57BL/6J and C57BL/6UofM mice experienced low fetal weight in response to *P. gingivalis*. However, low fetal weight appeared to be independent of the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A, IFN-γ or IL-4 in either substrain.

In *non-pregnant* C57BL/6UofM colonized with *P. gingivalis* the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A and IFN-γ were significantly higher than in sham-colonized females. In *pregnant* C57BL/6UofM such frequency of T cells producing IL-17A and IFN-γ was equal to sham-colonized. Similar findings occurred for the C57BL/6J mice. Thus, pregnancy appears to dampen the response to *P. gingivalis* mediated by IL-17A- and IFN-γ-producing $CD4^+$ T cells.

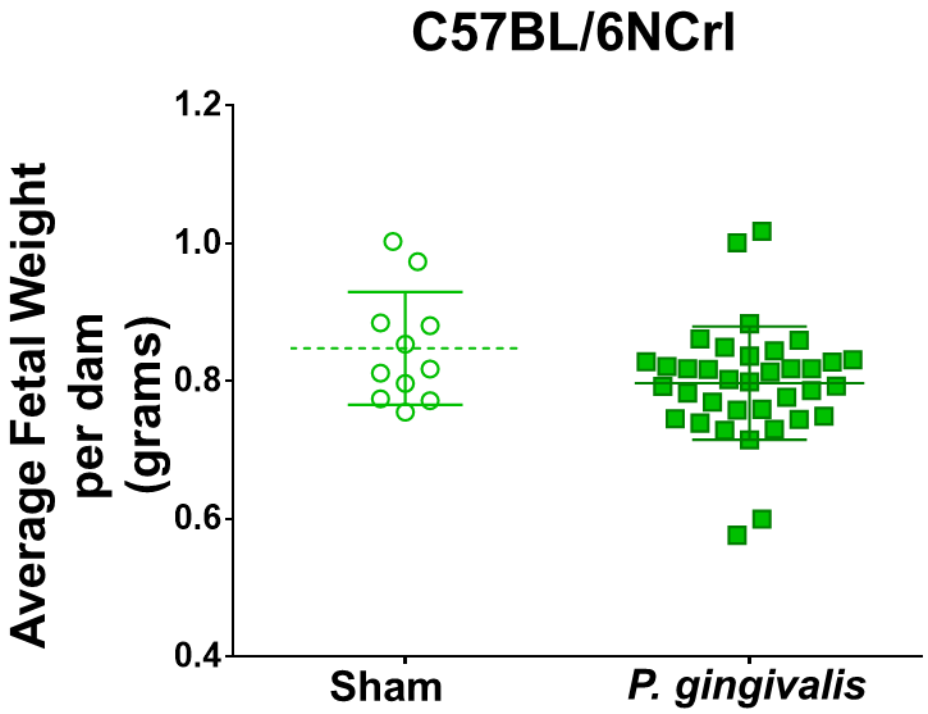
Given that the $CD4^+$ T cell response was independent of fetal weight in C57BL/6J and C57BL/6UofM mice, we tested a different C57BL/6 substrain that is prone to a heightened Th17 response. The Th17 response is induced by segmented filamentous bacterium (SFB) colonizing the ileum of C57BL/6NCrl, while C57BL/6J are SFB-negative. Other immunological differences between C57BL/6J and C57BL/6NCrl mice have been reported, but whether these differences are caused by genetics or microbiota has not been definitively evaluated (220, 221, 230).

Originally, we had hypothesized that an IFN-γ biased response to *P. gingivalis* in C57BL/6 background mice would be associated with low fetal weight

and an IL-17A response would not. The presence of elevated IL-17A in non-pregnant females of both C57BL/6UofM and C57BL/6J substrains lead us to wonder if instead, having more IL-17A in the C57BL/6NCrl mice would lead to an even greater reduction in fetal weight due to the inability to fully dampen excess IL-17A production during pregnancy.

C57BL/6NCrl mice Do Not Experience Low Fetal Weight in Response to Oral Colonization with *P. gingivalis*

Surprisingly, despite having the same overall genetic background as C57BL/6J mice or C57BL/6UofM, in C57BL/6NCrl mice fetal weight was the same in females orally colonized with *P. gingivalis* or with sham (Figure 13). Given that the C57BL/6 substrains that *did* experience low fetal weight had detectable *P. gingivalis* DNA in their placentas, we hypothesized that there would be an *absence* of *P. gingivalis* in the placentas of C57BL/6NCrl mice.



Parameter	Sham vs *P. gingivalis*
Fetal Weight	0.847 vs 0.797 g, p = 0.09
n sham dams	11
n P. gingivalis dams	36

Figure 13. Fetal Weight in C57BL/6NCrl Mice Orally Colonized with *P. gingivalis* was Similar to Controls. Fetuses were harvested on day E17 from dams that were orally colonized with *P. gingivalis* or sham only. After removal of the placenta, amniotic sac, and amniotic fluid the fetuses were weighed. Student's unpaired *t*-test with Welch's correction was used to compare the average fetal weight per dam and average fetal weight per group. Symbols (circles and squares) show average fetal weight per dam/litter. Experimental groups shown display average ± SD.

Fetal weight in the C57BL/6NCrl was Independent of *P. gingivalis* DNA in Placenta

We discovered that only 6.7% of the placentas were colonized with *P. gingivalis* in C57BL/6NCrl mice whereas 66% of placentas of C57BL/6J mice were colonized. Moreover, the fetal weight was independent of the amount of *P. gingivalis* in the placenta in C57BL/6NCrl dams (Figure 14). This indicates that there is an environmental or genetic difference that impairs the ability of *P. gingivalis* to colonize the placenta in this substrain of mice. We therefore hypothesized that in these dams there would be few *P. gingivalis*-specific T cells due to lack of *P. gingivalis* for local re-stimulation.

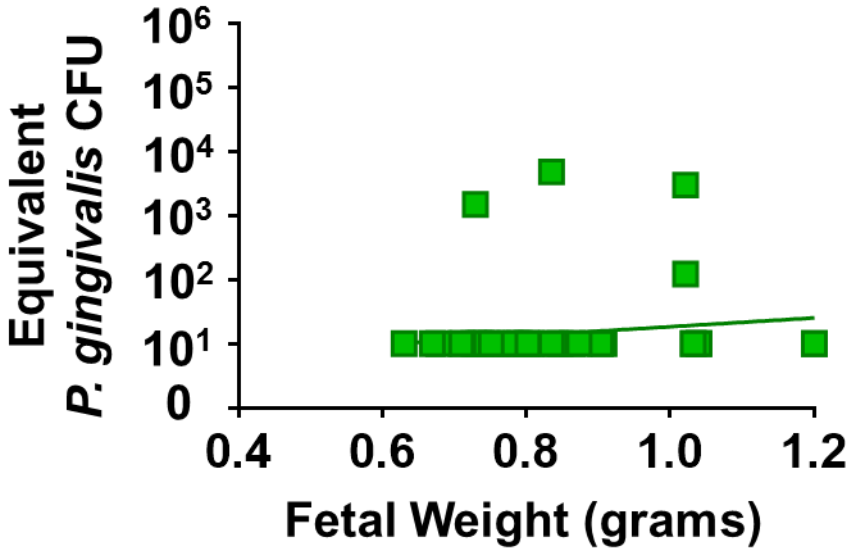


Figure 14. Amounts of *P. gingivalis* DNA in C57BL/6NCRL Placentas is Not Correlated with Reduced Fetal Weight. Nested qPCR with primers specific for a region spanning between the 16s and 23s rRNA genes of *P. gingivalis* was utilized. Fetal weight was determined after removal of amniotic fluid, sac, and placenta. Ng of *P. gingivalis* DNA per 250ng of placental DNA was determined using a standard curve generated from naïve mouse DNA combined with known amounts of *P. gingivalis* pure culture DNA. All samples for which no *P. gingivalis* DNA could be detected were set at the limit of detection, (0.07 pg or 10 CFU). Amounts of *P. gingivalis* DNA (measured in ng) were independent of fetal weight when assessed by Spearman's correlation. Each data point represents the weight one fetus (*x*) to *P. ginivalis* DNA in the corresponding placenta (*y*). Data was converted to equivalent CFU of *P. gingivalis* to demonstrate clinical relevance.

In *P. gingivalis* Colonized C57BL/6NCrl Pregnant Dams the Frequency of *P. gingivalis* specific CD4$^+$ Th cells Producing IL-17A, IFN-γ, or IL-4 in the CLN was Like Sham

The frequency of *P. gingivalis*-specific CD4$^+$ T cells producing IL-17A, IFN-γ, or IL-4 observed in *P. gingivalis* colonized C57BL/6NCrl pregnant females on day E17 was the same as that observed in sham colonized females (Figure 15, Right). Interestingly, there was an increased frequency of *P. gingivalis*-specific CD4$^+$ T cells producing IL-17A, IFN-γ, and IL-4 cytokines in non-pregnant females (Figure 15, Left). Thus, all three substrains of C57BL/6 mice (UofM, J and NCrl) have an increased frequency of *P. gingivalis*-specific CD4$^+$ T cells producing IL-17A when non-pregnant. The frequency of *P. gingivalis*-specific CD4$^+$ T cells producing IL-17A in non-pregnant females was similar across the three C57BL/6 substrains, when evaluated by a Mann-Whitney non-parametric test. C57BL/6J and C57BL/6NCrl were thus similar in their ability to produce IL-17A in response to oral colonization with *P. gingivalis*. Non-pregnant C57BL/6UofM and C57BL/6NCrl had also an increased frequency of *P. gingivalis*-specific CD4$^+$ T cells producing IFN-γ. The results in this substrain showing an increased production of IL-17A and IFN-γ in non-pregnant but not pregnant females thus further supports the concept that pregnancy dampens Th1 and Th17 responses.

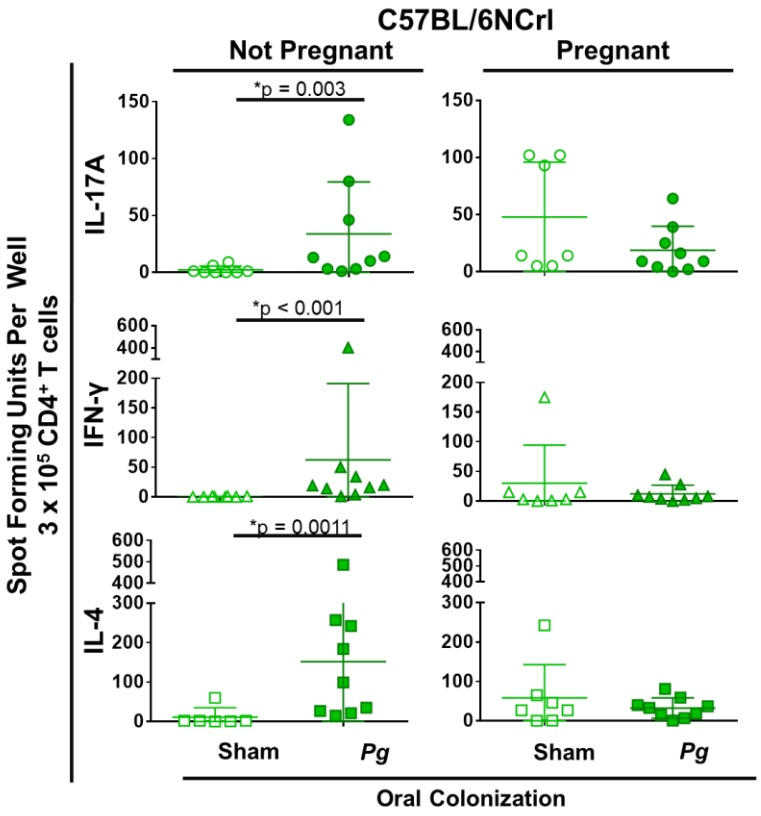


Figure 15. Non-pregnant C57BL/6NCrl Mice Orally Colonized with *P. gingivalis* Developed more *P. gingivalis*-specific $CD4^+$ T cells Producing IL-17A, IFN-γ, and IL-4 than Pregnant Mice. PaLN-CLN was harvested from dams that were orally colonized with *P. gingivalis* or sham only. $CD4^+$ T cells were purified and plated with irradiated naïve splenocytes for ELISpot to determine phenotype of cytokine output. After in vitro re-stimulation with whole *P. gingivalis*, Mann Whitney non-parametric test was used to compare frequency of $CD4^+$ T cells producing IL-17A, IFN-γ, and IL-4 in the *P. gingivalis* colonized versus sham females. Data is shown as median ± interquartile range.

In C57BL/6NCrl, when the average weight of each single litter is considered, there was a significant negative correlation between average fetal weight of that litter and the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ in the CLN of the dam producing that litter (Figure 16).

However, despite the impact that the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ had on average fetal weight of individual dams, the average fetal weight of all dams that were sham-colonized was the same as the average fetal weight of all dams that were *P. gingivalis*-orally colonized.

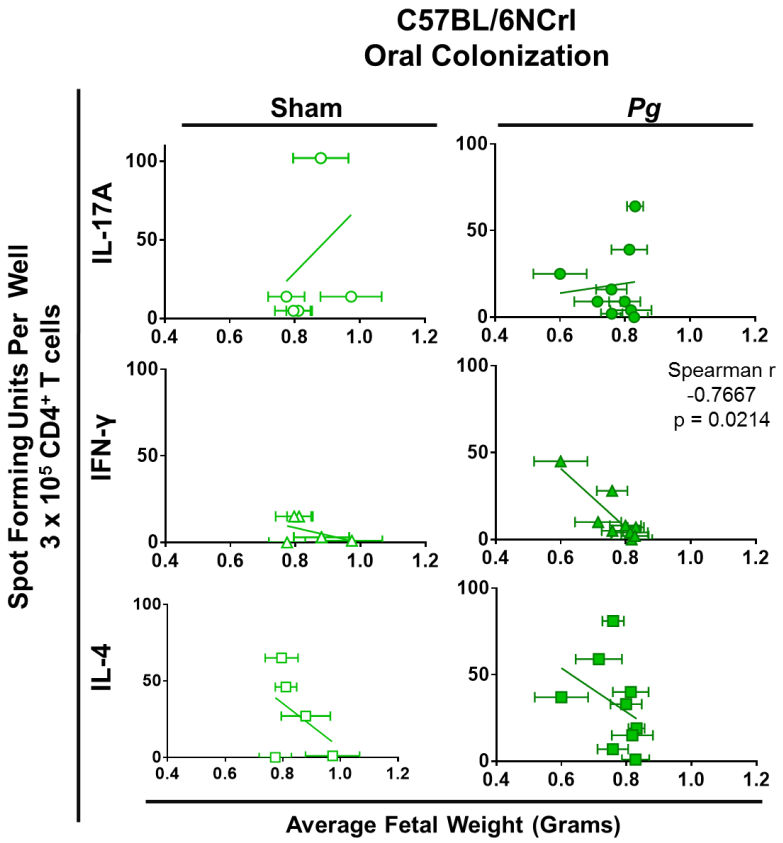


Figure 16. In C57BL/6NCrl Dams Orally Colonized with *P. gingivalis*, Lower Fetal Weight Correlated with Increased Frequency of *P. gingivalis*-specific $CD4^+$ T cells Producing IFN-γ. PaLN-CLN were harvested on day E17 from dams that were orally colonized with *P. gingivalis* or sham only. $CD4^+$ T cells were purified and plated with irradiated naïve splenocytes to determine phenotype of cytokine output. After removal of the placenta, amniotic sac, and amniotic fluid fetuses were individually weighed. Spearman's correlation was used to correlate the frequency of $CD4^+$ T cells producing IFN-γ in response to in vitro whole *P. gingivalis* re-stimulation and the average fetal weight ± SD in dams that had been orally colonized with *P. gingivalis* or sham.

Summary of C57BL/6NCrl in comparison to C57BL/6J and UoM

In stark contrast to the C57BL/6J and C57BL/6UofM subtrains, *P. gingivalis* in the placenta was independent offetal weight in C57BL/6NCrl mice. Additionally, far fewer placentas are colonized in C57BL/6NCrl mice as compared to C57BL/6J mice. In C57BL/6 background mice, it appears that *P. gingivalis* colonization of the placenta leads to low fetal weight, although whether *P. gingivalis* leads to low fetal weight directly or indirectly remains unclear. Similarly, if *P. gingivalis* is unable to colonize the placenta, C57BL/6 mice are protected from low fetal weight.

SFB colonization in C57BL/6UofM and C57BL/6NCrl mice

Non-pregnant C57BL/6UofM, C57BL/6J, and C57BL/6NCrl female mice all produced significant and equal amounts of IL-17A in their $CD4^+$ T cells in response to oral colonization with *P. gingivalis*. Intestinal SFB therefore did not appear to be necessary to induce *P. gingivalis*-specific Th17-type $CD4^+$ T cells in the oral cavity when measured in the CLN of C57BL/6J. In all three cases, pregnancy appears to dampen the IL-17A response such that sham and P*. gingivalis*-orally colonized females become similar.

It was verified that C57BL/6J and C57BL/6UofM mouse intestines did not harbor intestinal SFB in our experiments (Figure 17). The SFB-specific PCR amplicon band was consistently stronger in BALB/cAnNCrl than C57BL/6NCrl

mice when the same amount of starting DNA was used. SFB was not detected in any C57BL/6UofM mouse.

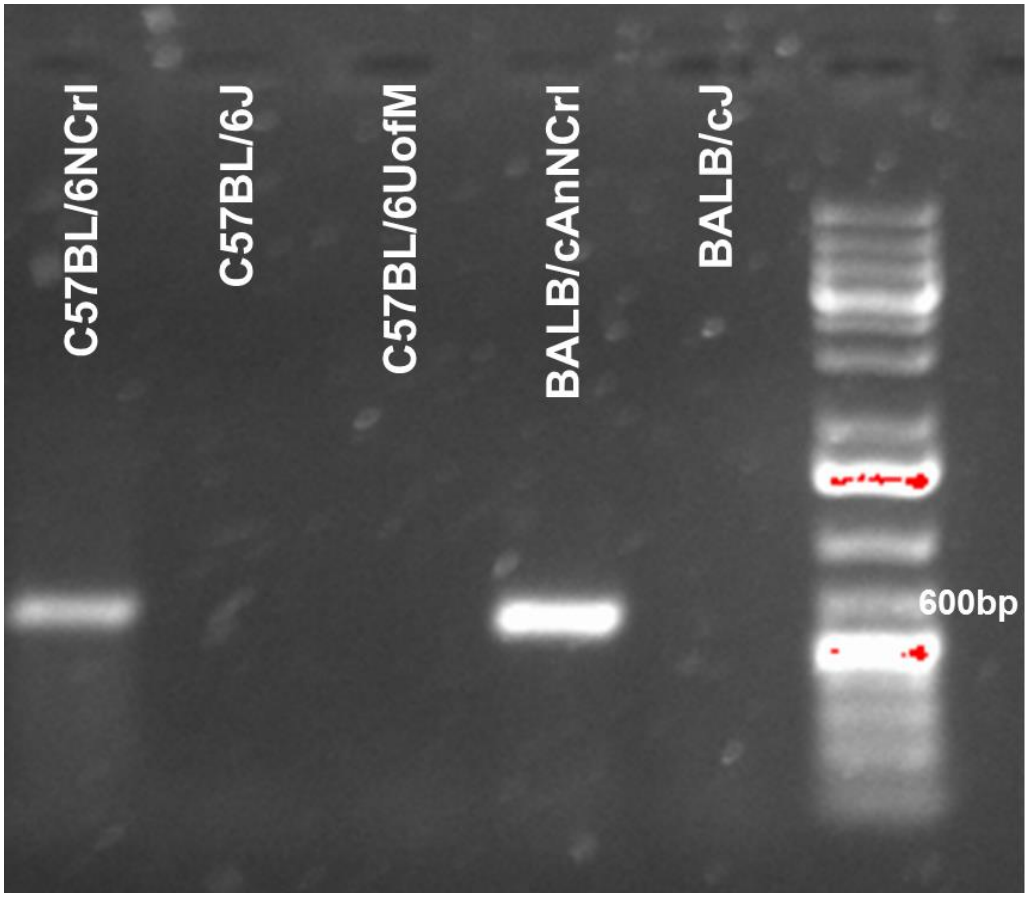
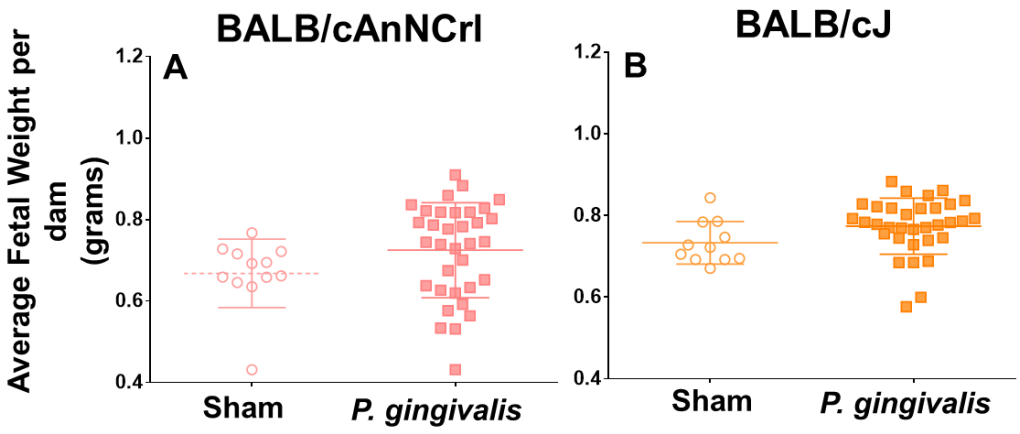


Figure 17. Only BALB/cAnNCrl and C57BL/6NCrl Mice Harbor SFB in their Intestines. Ilium intestinal sections were isolated from multiple mice from each substrain and digested for total DNA isolation. Conventional PCR was then performed on a standardized amount of total collected DNA to determine the presence or absence of SFB in each substrain, as described in (229).

Average Fetal Weight was Similar in BALB/cJ and BALB/cAnNCrl mice in Response to Oral Colonization with *P. gingivalis* or Sham

Considering there is a difference in fetal weight outcomes between C57BL/6J and C57BL/6NCrl mice, we also tested BALB/c mice from the same respective vendors. Regardless of vendor substrain, BALB/c mice had similar fetal weights whether orally colonized with *P. gingivalis* or sham (Figure 18 A, B).



Parameter	BALB/cAnNCrl Sham vs *P. gingivalis*	BALB/cJ Sham vs *P. gingivalis*
Fetal Weight	0.668 vs 0.725 g, p > 0.05	0.733 vs 0.774 g, p > 0.05
n sham dams	12	11
n *P. gingivalis* dams	34	33

Figure 18. Equal Fetal Weight in BALB/cAnNCrl and BALB/cJ Dams After Oral Colonization with *P. gingivalis*. Fetuses were harvested on day E17 from dams that were orally colonized with *P. gingivalis* or sham alone. After removal of the placenta, amniotic sac, and amniotic fluid fetuses were weighed. Student's unpaired *t*-test with Welch's correction was used to compare the average fetal weight per dam and average fetal weight per group in both vendor substrains of BALB/c after oral *P. gingivalis* colonization. Symbols (circles and squares) show average fetal weight per dam/litter ± SD. Experimental groups shown display average ± SD.

We next decided to evaluate the presence of *P. gingivalis* in the placentas of BALB/c mice. Given that C57BL/6J mice with *P. gingivalis* in their placentas had low fetal weight and C57BL/6NCrl mice without *P. gingivalis* in their placentas did not have low fetal weight, we predicted that we would not find *P. gingivalis* in the placentas of BALB/c mice.

In contrast, we found that 26% of BALB/cAnNCrl placentas and 50% of BALB/cJ placentas harbored detectable levels of *P. gingivalis*. In both vendor substrains of BALB/c mice, within individual dams, increasing amounts of *P. gingivalis* DNA in the placenta correlates with decreasing average fetal weight. However, the average combined fetal weight of all sham-colonized and *P. gingivalis*-orally-colonized BALB/c dams was the same. BALB/c mice are therefore resistant to significant fetal weight reduction as a consequence of *P. gingivalis* colonization of the placenta whereas the C57BL/6 mice are susceptible.

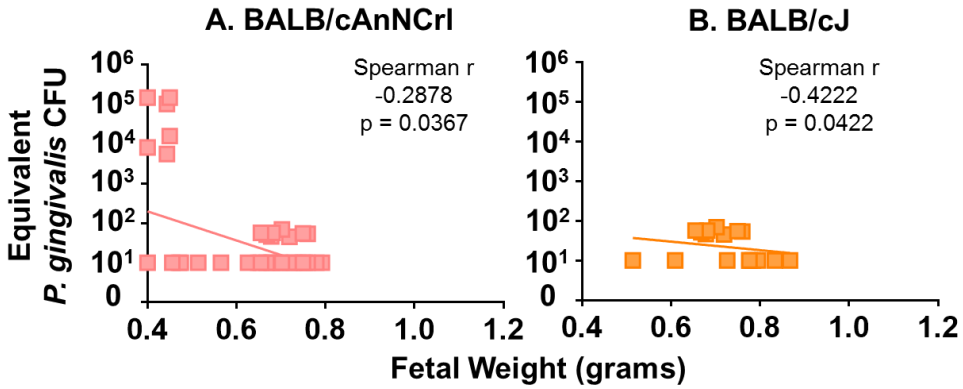


Figure 19. Increasing Amounts of *P. gingivalis* DNA in BALB/c Placentas is Correlated with Reduced Fetal Weight. Nested PCR with primers specific for an intergenic region between the 16s and 23s rRNA genes of *P. gingivalis* was utilized. Fetal weight was determined after removal of amniotic fluid, sac, and placenta. Ng of *P. gingivalis* DNA per 250ng of placental DNA was determined using a standard curve generated from naïve mouse DNA combined with known amounts of *P. gingivalis* pure culture DNA. All samples for which no *P. gingivalis* DNA could be detected were set at the limit of detection (0.07 pg or equivalent 10 CFU) to ensure the most rigorous analysis. Increasing amounts of *P. gingivalis* DNA (measured in ng) were compared with decreasing fetal weight by Spearman's correlation. Each data point represents the weight one fetus (*x*) to *P. ginivalis* DNA in the corresponding placenta (*y*). Data was converted to equivalent CFU of *P. gingivalis* to demonstrate clinical relevance.

Non-pregnant BALB/cJ females developed a greater frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A after oral colonization with *P. gingivalis* (Figure 20B). Non-pregnant BALB/CAnNCrl mice had a non-significant trend towards increased frequency of IL-17A producing *P. gingivalis*-specific $CD4^+$ T cells (Figure 20A = 0.082).

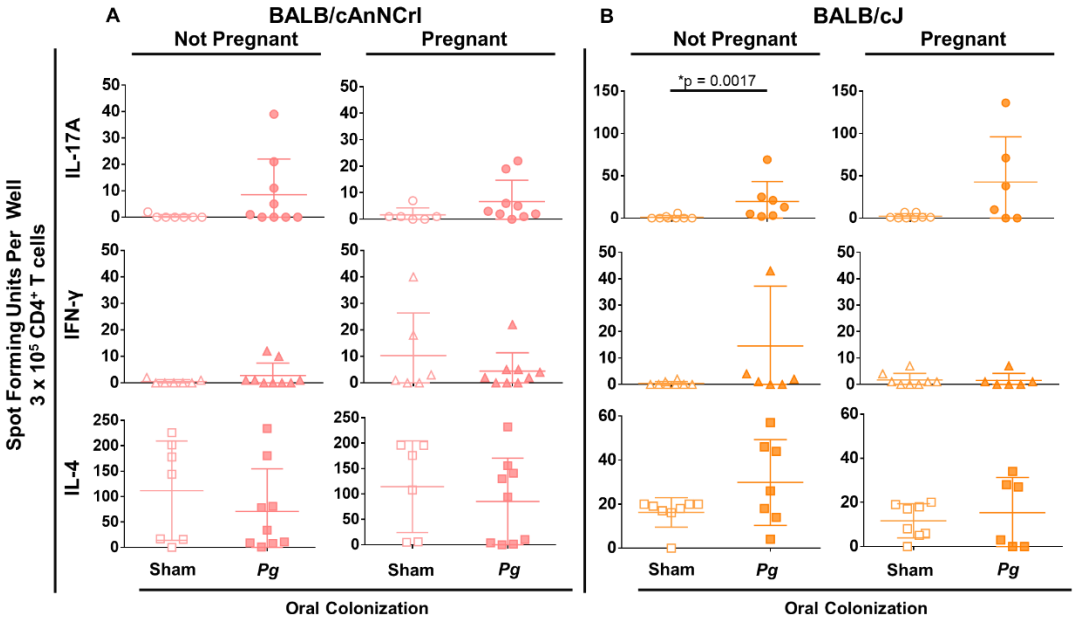


Figure 20. Frequency of CD4$^+$ T cells Producing IL-17A was Significantly Increased in Response to *P. gingivalis* in the PaLN-CLN of Non-pregnant BALB/cJ females. PaLN-CLN was harvested on predicted day E17 from dams that were orally colonized with *P. gingivalis* or sham only. CD4$^+$ T cells were purified and plated with irradiated naïve splenocytes for ELISpot to determine phenotype of T cell cytokine output. Mann Whitney non-parametric test was used to compare frequency of CD4$^+$ T cells producing IL-17A, IFN-γ, or IL-4 in the PaLN-CLN of BALB/females.

Decreased frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A was correlated with increasing fetal weight in sham colonized BALB/cAnNCrl females, but this association was possibly driven by small sample size (Figure 21A). Frequency of *P. gingivalis* $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 was independent of fetal weight in both vendor substrains of BALB/c mice after oral colonization with *P. gingivalis* (Figure 21A,B).

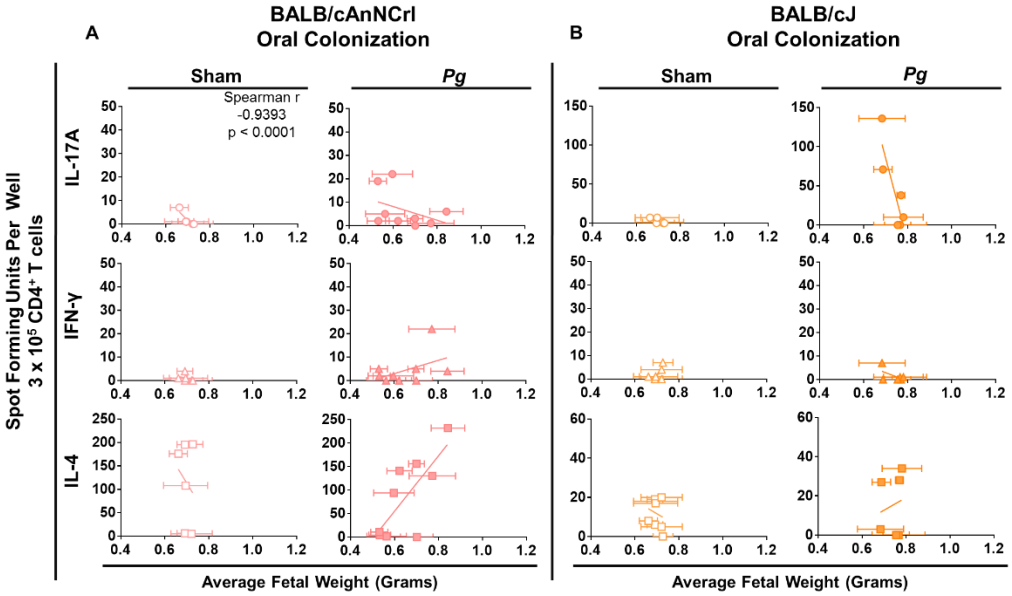


Figure 21. Fetal Weight was Independent of Frequency of $CD4^+$ T Cells Producing IL-17A, IFN-γ, or IL-4 in Response to *P. gingivalis* on Day E17 in the PaLN-CLN of BALB/c Dams. PaLN-CLN were harvested on day E17 from dams that were orally colonized with P. gingivalis or sham alone. CD4+ T cells were purified and plated with irradiated naïve splenocytes to determine phenotype of cytokine output. After removal of the placenta, amniotic sac, and amniotic fluid fetuses were individually weighed. Spearman's correlation was used to evaluate any potential correlation between average fetal weight ± SD and frequency of $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 in response to in vitro whole *P. gingivalis* re-stimulation.

Summary of Outcomes Related to Fetus

The following table summarizes the results for *P-gingivalis*-specific $CD4^+$ T cell phenotype, *P. gingivalis* presence in placenta, and fetal weight for each vendor substrain of C57BL/6 and BALB/c mouse used (Table 6).

Table 6. *P. gingivalis*-specific T Cell Phenotypes and Fetal Outcomes

Substrain	Fetal Weight: Sham versus *P. gingivalis* oral colonization	*P. gingivalis* DNA in placenta and Fetal Weight	Frequency of *P. gingivalis*-specific T cell producing IL-17A, IFN-γ or IL-4 after oral colonization with *P. gingivalis* ($p < 0.05$)
C57BL/6UofM	*0.909 vs 0.802 g $p < 0.0001$	Negative Correlation	**Non-pregnant**: ↑ IL-17A and IFN-γ **Pregnant**: ↑ IL-4
C57BL/6J	*0.900 vs 0.722 g $p < 0.0001$	Negative Correlation	**Non-pregnant**: ↑ IL-17 **Pregnant:** Not different
C57BL/6NCrl	0.847 vs 0.797 g $p = 0.09$	Not Correlated	**Non-pregnant**: ↑IL-17A, IFN-γ, and IL-4 **Pregnant**: Not different
BALB/cJ	0.773 vs 0.774 g $p > 0.05$	Negative Correlation	**Non-pregnant**: ↑IL-17A **Pregnant**: Not different
BALB/cAnNCrl	0.668 vs 0.725 g $p > 0.05$	Negative Correlation	**Non-Pregnant**: Trend ↑IL-17A) **Pregnant**: Not different

Summary of Outcomes Related to Dam

The weight of *P. gingivalis*-colonized non-pregnant females or of dams after uterine excision were similar to sham-colonized females in all strains and substrains. Additionally, there was no difference in embryo lethality or litter size between any *P. gingivalis*- and sham-colonized groups. This suggests that *P. gingivalis* does not play a role in late term spontaneous abortion in this model.

The rate at which female mice with observed copulation plugs were pregnant at expected day 17 of gestation versus those females that were ultimately not pregnant was similar across all three C57BL/6 substrains, and between any sham or *P. gingivalis*-colonized C57BL/6 mice. The rate at which females with observed copulation plugs were pregnant versus not pregnant at expected day 17 of gestation was also similar between the two BALB/c substrains, or between any sham versus *P. gingivalis*-colonized BALB/c mice. This comparison is underpowered because to detect a difference in pregnancy rate of 20% with 80% Power using a chi-square test at the 0.05 alpha level we would have needed 104 C57BL/6UofM dams. In the C57BL/6NCrl to detect a 25% pregnancy rate difference we would have needed 67 dams per group. For other strain and substrains the number of dams needed would have been even higher as the difference in pregnancy rate was smaller.

In *P. gingivalis*-colonized mice only, there was a significant difference in pregnancy rate between the C57BL/6 mice and BALB/c mice ($p = 0.0179$). However, also in *P. gingivalis*-colonized mice only, the rate of pregnancy was

similar among all three C57BL/6 substrains or between the two the BALB/c substrains (Table 7).

SFB colonization of the ileum was as expected. Mice from Charles River Laboratory harbored SFB whereas mice from Jackson Laboratory did not. C57BL6/UofM, which were derived from Jackson mice, also did not harbor SFB (Figure 17).

Table 7. Rate of Pregnancy and Average Litter Size in Females with Observed Copulation Plugs

Substrain	Ratio Non-Pregnant to Pregnant		Average Litter Size	SFB Colonization of Ileum
	Sham	*P. gingivalis*-colonized	Sham -vs- *P. gingivalis*-colonized	
C57BL/6UofM	11:11 (50%)	16:36 (70%)	7 – 8	No
C57BL/6J	9:16 (64%)	15:31 (67%)	8 – 8	No
C57BL/6NCrl	16:11 (41%)	19: 36 (66%)	8 – 8	Yes
BALB/cJ	11:11 (50%)	38:33 (46%)	7 – 7	No
BALB/cAnNCRL	14:12 (46%)	36:34 (50%)	6 – 8	Yes

Discussion

We hypothesized that increased frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ in the PaLN-CLN would correlate with decreasing fetal weight in C57BL/6J mice. In other words, we hypothesized that we could model the host response to periodontal inflammation attributable to *P. gingivalis*, and explain low birth weights based on the biology of responding T cells. Our data generally did not support our hypothesis of a correlation between *P. gingivalis*-specific $CD4^+$ T cell response and low fetal weight.

Low Fetal Weight Phenotype in C57BL/6UofM and C57BL/6J mice is Independent of Frequency of *P. gingivalis*-specific $CD4^+$ T cells Producing IFN-γ, IL-17A, or IL-4.

Frequency of recirculating memory *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ or IL-17A in PaLN-CLN on day E17 of gestation was the same in *P. gingivalis*-colonized C57BL/6J or C57BL/6UofM dams as compared to sham-colonized. Low fetal weight in C57BL/6J or C57BL/6UofM mice colonized with *P. gingivalis* was independent of the frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ, IL-17A or IL-4. These were the only two substrains that experienced fetal weight reduction in response to oral colonization with *P. gingivalis*.

We considered whether the test of correlation between fetal weight and frequency of $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4 was appropriately powered. In *P. gingivalis*-colonized C57BL/6J mice, the non-significant Spearman *r* values were 0.1725 for IL-17A, 0.3627 for IFN-γ, and 0.1783 for IL-4. In *P. gingivalis* colonized C57BL/6UofM mice the non-significant Spearman's *r* values were -0.01722 for IL-17A, 0.112 for IFN-γ, and -0.1216 for IL-4. To achieve 80% Power at alpha = 0.05 for IFN-γ with those correlation values would require a n of over 50 for C57BL/6J to over 500 mice for C57BL/6UofM. Importantly, Spearman's correlation measures the strength of association between two variables, with a value of 1 or negative 1 indicating a perfect positive or negative correlation respectively. In conclusion, testing 500 mice for the sake of reaching sufficient Power for a weak correlation would not be a good use of resources.

The lack of ability to detect a significant correlation between fetal weight and frequency of $CD4^+$ T cells producing IL-17A, IFN-γ, or IL-4, as well as the weak, positive *r* values for this non-significant correlation, suggests that recirculating *P. gingivalis*-specific $CD4^+$ T cells in the PaLN-CLN of C57BL/6J or C57BL/6UofM mice during pregnancy do not drive the low fetal weight phenotype.

Alternative potential explanations for this result are: i) that recirculating *P. gingivalis*-specific $CD4^+$ T cells in CLN do not represent *P. gingivalis*-specific

$CD4^+$ T cells present in the uterus and/or placenta and ii) that the frequency of such T cells in PaLN is too low.

PaLN which drain the reproductive track should contain recirculating *P. gingivalis*-specific $CD4^+$ T cells from the uterine and placental area, given the presence of *P. gingivalis* antigens in the placenta. When combined for ELISpot assays, CLN and PaLN produced on average 3×10^6-8×10^6 $CD4^+$ T cells per mouse but PaLN $CD4^+$ T cells were proportionally lower than those isolated from the CLN.

The lack of ability to detect a correlation between fetal weight and $CD4^+$ T cell response could also be related to timing between initial oral inoculation and time of conception. Our time course was designed such that periodontitis, measured by alveolar bone destruction, would be present in these mice at the time of conception. However, we have also observed in related studies that detectable numbers of *P. gingivalis*-specific $CD4^+$ T cells in the CLN decrease over time in a mouse that is chronically infected with oral *P. gingivalis* (224). Since *P. gingivalis* invades the tissues it is likely that its antigens are being presented by professional antigen presenting cells (APCs) in the tissue to memory CD4+ T cells, as opposed to presentation by APC to naïve CD4+T cells in the draining lymph nodes. As chronic infection progresses in mice this antigen presentation would keep memory $CD4^+$ T cells within the tissues. Therefore, there may have simply been too few recirculating *P. gingivalis*-specific $CD4^+$ T cells left in CLN and PaLN to obtain sufficient numbers of *P. gingivalis*-specific

$CD4^+$ T cells to accurately phenotype them during gestation in the chronic mucosal infection model. Unfortunately, pooled placentas or uterine tissue did not yield sufficient numbers of *P. gingivalis*-specific $CD4^+$ T cells of a high enough purity to conduct the ELISpot even when the placentas contained within the uterus were positive for *P. gingivalis* DNA at E17 of gestation. It might have been possible to detect higher numbers of *P. gingivalis*-specific $CD4^+$ T cells in PaLN-CLN with a shorter time course. However, we do not currently know whether fetal weight reduction would be observed with a shorter *P. gingivalis* inoculation time course or if prolonged chronic oral infection is required to induce low fetal weight. It remains a question that future research may be able to answer.

All Non-Pregnant Females Harbor *P. gingivalis*-specific $CD4^+$ T cells Producing IL-17A after Oral Colonization with *P. gingivalis*

P. gingivalis colonized non-pregnant females from all substrains of C57BL/6 mice and BALB/cJ had a significantly greater frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A in their PaLN-CLN than sham-colonized non-pregnant mice. BALB/cAnNCrl mice demonstrated a similar non-significant trend. Non-pregnant C57BL/6NCrl mice had the same frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A in their PaLN-CLN as C57BL/6J mice. Surprisingly, this means that female mice from strains lacking intestinal SFB still mounted the same magnitude of Th17-biased response against oral *P. gingivalis.*

Definitively then, while essential to a submucosal Th17 response in the small intestinal lamina propria, a lack of intestinal SFB does not seem to impair the ability of non-pregnant C57BL/6UofM or J mice to mount a Th17-specific response to oral *P. gingivalis*. Some *P. gingivalis* is always swallowed by the mice during oral gavage. Thus, the possibility of a role for SFB-driven bystander activation of *P. gingivalis*-specific intestinal Th17 cells remains for protection against *P. gingivalis*-mediated low fetal weight in C57BL/6NCrl mice. Low fetal weight in C57BL/6NCrl mice may also be prevented by a placental microbiome that is not permissive to *P. gingivalis* colonization.

Even though both non-pregnant BALB/c and C57BL/6 mice had an increased frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IL-17A, only non-pregnant C57BL/6UofM and C57BL/6NCrl females developed a significantly higher frequency of *P. gingivalis*-specific $CD4^+$ T cells producing IFN-γ. The C57BL/6J substrain was expected to have the highest IFN-γ response to oral *P. gingivalis* because they are historically considered Th1-biased. Surprisingly, C57BL/6J was the only tested C57BL/6 substrain that did not have an increased frequency of IFN-γ *P. gingivalis*-specific $CD4^+$ T cells in non-pregnant mice in response to oral colonization with *P. gingivalis*.

Potential Explanations for the Discrepancy Between Non-Pregnant and Pregnant Females in Response to *P. gingivalis*

Non-pregnant and pregnant female mice were harvested on the same equivalent time point in the experimental timeline, ruling out that a temporal difference is responsible for the difference in cytokine output. Non-pregnant and pregnant female mice both had observable copulation plugs. The difference between the two conditions is whether fetuses were present in the uterus at expected embryonic day 17 (E17).

It is formally possible that pregnancy dampens or attenuates the adaptive immune response against *P. gingivalis*, resulting in a non-significant difference in cytokines between sham and *P. gingivalis* colonized females. Pregnancy has been seen to dampen the adaptive immune response in animal models of autoimmunity, as discussed previously.

One alternative explanation is that cytokines being produced by *P. gingivalis*-specific $CD4^+$ T cells were involved in failure of implantation or early fetal abortion. Strong anti-*P. gingivalis* adaptive response may have prevented implantation resulting in lack of pregnancies. The lack of a difference in the successful copulation plug-to-pregnancy rates observed within or across C57BL/6 and BALB/c substrains would suggest against this possibility, but our study population was too small to definitively rule this possibility out. Staining the uterus of non-pregnant females to identify implantation sites indicating early spontaneous abortion could potentially help resolve this issue in a future study.

Given that the difference in fetal weight outcomes cannot be explained by production of IL-17A, IFN-γ, or IL-4 by recirculating *P. gingivalis*-specific memory $CD4^+$ T cells, it becomes necessary to evaluate other potential causes for low fetal weight only in specific mouse substrains induced by oral colonization with *P. gingivalis*. The correlation of *P. gingivalis* in the placental to low fetal weight in C57BL/6J and C57BL/6UofM mice, but not C57BL/6NCrl mice suggests a role for the placental, oral, or intestinal microbiome in preventing *P. gingivalis* colonization of the placental and subsequent *P. gingivalis*-mediated fetal weight outcomes. The lack of low fetal weight in BALB/c mice despite comparatively high amounts of *P. gingivalis* DNA in their placentas suggests a genetic component of *P. gingivalis*-mediated fetal weight outcomes, perhaps driven by differences in innate immunity or components of the adaptive immune system other than $CD4^+$ T cells.

Microbial Communities and *P. gingivalis*-mediated Low Fetal Weight

Reducing *P. gingivalis* from the periodontal pocket in SRP trials fails to reduce the risk of APOs (125, 136, 139, 231). Therefore, *P. gingivalis* in the oral cavity *alone* does not appear to be a major driver of periodontitis-associated APOs, as that would require an immune response of significant magnitude to produce enough systemic cytokines to influence the fetoplacental unit. For this reason, we needed to evaluate the presence of periodontal pathogens in the

placenta, and the potential that periodontal pathogens in the placenta could lead to local re-stimulation of memory $CD4^+$ T cells.

We determined that mere presence of *P. gingivalis* in the placenta, as proxy measured by amount of *P. gingivalis* DNA, was less useful in predicting whether low fetal weight would occur in C57BL/6J mice than the amount of *P. gingivalis* present. This mimics the situation in the oral cavity, where *P. gingivalis* is normally found at low levels in the orally healthy individual and significantly higher levels in the periodontally diseased individual.

P. gingivalis in the C57BL/6 placenta

Sustained oral colonization with *P. gingivalis* of C57BL/6J and C57BL/6UfoM for six weeks leads to low fetal weight. After embryo implantation and placental development, *P. gingivalis* can be detected in the established placenta but not in uterine tissues or blood at E17. The amount of *P. gingivalis* DNA within 250 ng of placental DNA increased as fetal weight decreased. This observation is not an artifact because the amount of starting total DNA in each qPCR sample was identical. In C57BL/6NCrl, the strain that did not experience low fetal weight, fetal weight was independent of amount of *P. gingivalis* DNA and fetal weight. Additionally, far fewer placentas were colonized with *P. gingivalis* in C57BL/6NCrl as compared to C57BL/6J mice.

Does either the C57BL/6NCrl Microbiome or SFB Have a Role in Pregnancy Outcomes?

There are three major explanations for this difference in placental colonization between C57BL/6J and C57BL/6UfoM versus the C57BL/6NCrl mice. First, it is possible that the non-*P. gingivalis*-specific SFB-mediated Th17-biased predisposition of the C57BL/6NCrl mouse may be more effective at preventing *P. gingivalis* colonization or facilitating *P. gingivalis* clearance. Second, *P. gingivalis* may be less able to invade and colonize the placental microbiomes of the C57BL/6NCrl mouse as compared to other substrains. SFB or the intestinal Th17 cell response it induces may be involved in protection against *P. gingivalis*-mediated low fetal weight, although it should not be assumed that SFB is the only important microbial difference between the C57BL/6 substrains. Third, the potential environmental niche for *P. gingivalis* within the microbial community could be already occupied by another bacterium or the niche could be entirely absent in C57BL/6NCrl placental microbiomes. There could even be bacterial species that actively antagonize *P. gingivalis*.

Despite the absence of SFB, C57BL/6UofM and J mouse $CD4^+$ T cells still produce IL-17A in response to oral *P. gingivalis*. Intestinal SFB colonization in C57BL/6NCrl and the consequent intestinal Th17 cells may protect against *P. gingivalis*-mediated low fetal weight by preventing *P. gingivalis* colonization of the placenta. Once stimulated by swallowed *P. gingivalis,* the intestinal memory *P. gingivalis*-specific $CD4^+$ T cells, rather than the oral ones, could recirculate and

eliminate potential *P. gingivalis* colonizing the C57BL/6NCrl placenta. This hypothesis is supported by the observation that *P. gingivalis* is found only in 6% of C57BL/6NCrl placentas as compared to 66% of C57BL/6J placentas.

Potential Alternative Mechanisms of *P. gingivalis*-mediated Low Fetal Weight

P. gingivalis colonizing the placenta is not entirely the reason for low fetal weight. Despite the presence of *P. gingivalis* in placentas in both BALB/c substrains, fetal weight was similar in *P. gingivalis*- and sham- colonized mice. Perhaps as periodontal pathogens such as *P. gingivalis* are necessary but not sufficient to induce periodontitis, it is plausible that species like *P. gingivalis* are necessary but not sufficient to induce APOs. Given that the adaptive, *P. gingivalis*-specific $CD4^+$ T cell response does not contribute to *P. gingivalis*-mediated low fetal weight susceptibility, it is reasonable to speculate that the difference in fetal weight outcomes in BALB/cJ and BALB/cAnNCrl versus C57BL/6J and C57BL/6UfoM can be attributed in part to variation in the innate immune response or other parts of the adaptive immune response.

It is likely that interactions between genetically determined variations in immunity and the environment determine susceptibility to infection and inflammation-induced APOS in mice, and probably also in humans. C57BL/6NCrl mice represent the intersection between genetics and microbiome

in determining susceptibility to low fetal weight in response to oral colonization with *P. gingivalis*. Although belonging to the same H-2 haplotype, C57BL/6N mice have a different inflammatory phenotype than C57BL/6J mice. Whether that difference is driven by genetic drift or differences in microbiota, or a combination of both, is still an open to debate. While SFB in C57BL/6N shows that the microbiota can alter the immune response, murine strain variations in the immune phenotype may also affect the populations of microorganisms that are capable of permanently colonizing the different strains of BALB/c or C57BL/6 mice and substrains of each.

Alternative Sources of IFN-γ from Innate and Adaptive Immunity

Our hypothesis focused on IFN-γ producing $CD4^+$ T cells specific for *P. gingivalis* because increasing amounts of systemic IFN-γ have been correlated with low fetal weight in previous animal studies. However, $CD4^+$ T cells producing IFN-γ may be specific for other species reacting to the presence of the keystone pathogen *P. gingivalis* or to the changes it induces in local nutrient availability, and not *P. gingivalis* itself. It is also possible that the source of elevated IFN-γ is not $CD4^+$ T cells. $CD8^+$ cytotoxic T cells, mucosal epithelial cells, natural killer and natural killer T cells can also produce IFN-γ in significant amounts. While excessive inflammation has been associated with APOs, some production of IFN-γ by uterine natural killer cells (uNK cells) is necessary for murine implantation (97, 232).

IL-10 Modulates the IFN-γ Response and can be Produced by Adaptive Tregs or Innate Cells of the Female Reproductive Track

The “increase” in Th1-associated cytokines detected historically may be a by-product of a decrease in Th2- and/or Th17-associated cytokines, or even failure of Treg to control inflammation.

IL-10 has been associated with both Th2 and Treg cells. The role of $CD4^+$ T cell production of IL-10, which may be involved in protection against low fetal weight, was not evaluated in these experiments. This is because in pilot experiments we detected high frequency of irradiated splenocytes producing IL-10. The frequency of irradiated splenocytes plus $CD4^+$ T cells from PaLN-CLN producing IL-10 from sham- or *P. gingivalis*-colonized mice was even higher than splenocytes alone and not different from one another. This finding prevented the determination of the true signal of IL-10 from $CD4^+$ T cells specific for *P. gingivalis* via ELISpot. Simply measuring systemic IL-10 would not suffice because, like IFN-γ, it can be produced by cells other than $CD4^+$ T cells. IL-10 can be produced by uterine natural killer cells and placental trophoblasts for example. It is especially worth noticing that uterine natural killer cells produce IFN-γ to assist with implantation, but also produce IL-10 to assist with fetal tolerance. Uterine natural killer cells can thus be pro- and anti-inflammatory, and both roles are important for normal pregnancy (232-234).

Tregs have been implicated in fetal tolerance and maintenance of pregnancy (97). We determined that pregnancy dampens the cytokine response as compared to non-pregnant females at the same time point, which suggests the involvement of Tregs and/or Treg-associated cytokines. Even though Tregs are known to produce IL-10, Tregs are usually identified by the expression of transcription factor FoxP3 rather than specific cytokines, which makes ELISpot not viable for detecting Tregs. Alternative techniques such as MCH class II tetramer staining or fate-tracking mice that express fluorescent proteins in Treg cells may be useful in determining whether adaptive Treg or IL-10 producing Treg/Th2 cells are involved in protection against *P. gingivalis*-mediated low fetal weight. We may need to consider the balance of Tregs to Th1 and Th17 cells to determine the mechanism of *P. gingivalis*-mediated low fetal weight.

Innate Immunity: *P. gingivalis* Modulates Complement and Toll-like Receptor Response

The actions of *P. gingivalis* on complement and toll-like receptors demonstrates one way the microbiome and host immunity can interact in a manner that is favorable to growth of the microorganism. *P. gingivalis*-mediated low fetal weight in BALB/c versus C57BL/6 may depend on differences in complement and toll-like receptor regulation and function.

The complement system always operates at a low, steady level of activation (235). There are three pathways that can activate complement to

provide host defense against microbial pathogens. Complement activation is tightly controlled by both soluble and membrane bound inhibitors. Complement regulation and some degree of complement deposition and activation occurs at the placental interface during normal pregnancy. APOs such as PLBW have been associated with excessive or misdirected complement activation, as demonstrated by women inherited or acquired complement disorders. Most inherited gene disorders in complement regulatory proteins that increase the risk of APOs are loss of function mutations, such as mutations in C4b binding protein. Human C3 deficiency leads to infertility in women because C3 is important in trophoblast invasion of the decidua and endometrial blood vessels. C1q knock-out mice have abnormal placentas, LBW, and demonstrate preeclampsia-like hypertension. Placental ischemia and hypertension in rats has been mechanistically linked to activation of C3a and C5a. Miscarriage events have been associated with hypocomplementia (235). Alternatively, gain of function in complement effector molecules has not been linked to APOs. Differences in C3b deposition in the alternative complement pathway have been reported between C57BL/6 and BALB/c mice, and has been proposed as a mechanism to explain differences in innate response to infection between these two strains (236).

P. gingivalis has several virulence factors that can manipulate complement in ways that promote slight inflammation which ultimately provides nutrients to favor its own growth. For example, gingipains can inhibit complement activation by degrading C3, the central complement component.

Arg-specific gingipains can bind the inhibitory C4b complement binding protein, which results in inhibition of the complement cascade and serum-induced killing (237).

Toll-like receptors (TLRs) are pattern recognition receptors and part of the microbial recognition system. They are considered innate immunity receptors because they are invariant. Most cells in the body express at least some TLRs, including gingival epithelial cells and the female reproductive tract (238). Placental trophoblasts express TLR2 and TLR4, and can express other TLRs (239).

TLRs recognize microbe associated molecular patterns (MAMPs). MAMPs are otherwise known as pathogen associated molecular patterns (PAMPs). For example, TLR4 recognizes lipopolysaccharide (LPS), TLR9 recognizes bacterial DNA, and TLR2 recognizes lipoteichoic acid (240). TLRs have been implicated in both the pathogenesis of periodontitis and APOs (238, 239, 241-243). For example, polymorphisms in TLR4 have been reported to be positively associated with both PTB and periodontitis (238, 244, 245). Reduced expression of TLR4 in endothelial cells of terminal villi has been associated with preeclampsia (246). Combined oral inoculation with *Campylobacter rectus* and *P. gingivalis* leads to a trend of increased TLR4 expression in murine placental labyrinth tissue (153, 247). Chorionic tissues from high risk pregnancies react to *P. gingivalis* LPS by releasing IL-6 and IL-8 in a TLR2-dependent manner (248). In contrast, tissue explants from normal, full term fetal membrane showed an

increase in mRNA for TLR7 after incubation with *P. gingivalis* (249). Interestingly, different bacteria have been shown to induce increases in different toll-like receptor mRNA in full term fetal membrane explants (249), indicating species-specificity in the TLR response. Changes in mRNA expression for TLRs indicate that *P. gingivalis* can induce an innate host immune response in placental tissue. If *P. gingivalis* induces APOs through changing the placental microbiome, changes in mRNA expression for TLRs may reflect changes in abundance of bacterial species including but not limited to *P. gingivalis*.

Strain-specific differences in TLR response to pathogens has been reported in BALB/c versus C57BL/6 mice. For example, in response to *Ureaplasma parvum*, BALB/cJ mice develop chorioamnionitis and fetal inflammatory response syndrome-like pathology in conjunction with an increased expression of TLRs 1, 2, 6 in infected placentas. In contrast, expression of these TLRs in response to *Ureaplasma parvum* in unaffected C57BL/6J mice was unchanged or down-regulated (240).

Even in naïve mice there appears to be strain-specific differences in TLR expression between BALB/c and C57BL/6 mice. Dendritic cells from spleens of naïve C57BL/6NCrl mice preferentially expressed TLR9 mRNA. In contrast, BALB/cAnNCrl mice preferentially expressed TLR2, 4, 5, and 6 mRNAs. C57BL/6NCrl dendritic cells respond to ligands for TLR4, TLR2, and TLR9 by producing IL-12p40 whereas BALB/cAnNCrl produced monocyte chemoattractant protein 1 (MCP-1) (250). It is thus plausible that differences in

P. gingivalis-mediated fetal weight outcomes between BALB/c and C57BL/6 mice may be driven by differences in interactions between either complement or toll-like receptors and *P. gingivalis*.

P. gingivalis LPS is rather unique, as it contains multiple lipid A species that functionally interact with both TLR2 and TLR4 (150, 251-253). Lipid A 1- and 4'- phosphatases generate LPS structures that function as antagonists of TLR4 allowing *P. gingivalis* to escape TLR4-mediated antimicrobial functions, such as induction of antimicrobial peptides (254). *P. gingivalis* releases LPS-bearing membrane vesicles that can inhibit TLR4 responses against other bacteria in the same biofilm (254). The TLR system can compensate for the action of *P. gingivalis* on TLR4 by sensing *P. gingivalis* primarily through TLR2. *P gingivalis* therefore has evolved different mechanisms to evade TLR2 and TLR4 signaling in neutrophils and macrophages (251).

P. gingivalis and Neutrophils

P. gingivalis can inhibit neutrophil migration, apoptosis and neutrophil killing functions, while activating neutrophils to release pro-inflammatory mediators (31, 255). All three gingipain enzymes can lead to complement inactivation (251). Gingipains can clip complement C5 protein to generate the anaphylatoxin C5a and destroy C5b. Binding of C5a to C5aReceptor induces ubiquitination of MyD88 and generation of phosphatidylinositol-3-kinase (PI3K) in neutrophils. The TLR2-dependent recognition of *P. gingivalis* and of bystander

microorganisms is therefore inhibited by lack of MyD88 signaling. The generation of PI3K inhibits actin polymerization and therefore phagocytosis in neutrophils, but most importantly it promotes periodontal inflammation via TNF, IL-1β, IL-17 and IL-6 resulting in a dysbiotic microbiota (31, 150, 251). This two-pronged mechanism uncouples the synergistic effect of C5aR and TLR2, resulting in inhibition of bacterial immune clearance by neutrophils but favoring a pro-inflammatory environment. The pro-inflammatory environment generates a nutritionally favorable state for *P. gingivalis* and the bystander microbiota.

P. gingivalis and Macrophages.

P. gingivalis fimbriae binds complement receptor-3 (CR3) activating phagocytosis in macrophages while preventing TLR2-induced IL-12 production. CR3-mediated phagocytosis, however, fails to kill *P. gingivalis* because of the inhibition of inducible nitric oxide synthase (iNOS) mediated by the increased cyclic adenosine monophosphate (cAMP) produced secondary to Arg-gingipain-dependent C5a-C5aR binding. Increases in cAMP and subsequent increases in protein kinase A (PKA) further impairs nitric-oxide dependent killing of *P. gingivalis* in macrophages (150, 251, 254). Fimbriae also bind to CXCR4 and TLR2 to induce cAMP-dependent PKA signaling. cAMP impairs TLR2-driven nitric oxide dependent killing of *P. gingivalis* in macrophages (150, 254). Maximum cAMP and therefore maximum inhibition of iNOS is achieved when both C5aR-TLR2 and CXCR4-TLR2 pathways are activated by *P. gingivalis*

(254). The resulting scenario is persistence of live *P. gingivalis* in endocytic vacuoles in a sustained inflammatory environment which may lead to inflammation-induced APOs (251, 254).

While *P. gingivalis*-mediated C5aR-TLR2 and CXCR4-TLR2 crosstalk has been heavily studied in periodontitis models, very little has been reported on modulation of TLRs and complement by *P. gingivalis* in the placenta. Even less has been reported on *P. gingivalis*-mediated manipulation of complement in placentas. Since it has now been established that *P. gingivalis* can be detected in placentas associated with APOs in both humans and mice, studies of *P. gingivalis* subversion of TLR-complement cross talk should focus on placental as well as gingival tissue. These studies should consider that *P. gingivalis* may not have the same effect on TLR and complement in BALB/c as compared to C57BL/6 mice.

Epigenetics: A Bridge Between Genetics and Environment

Epigenetic changes result in changes in the availability of DNA for transcription and translation into mRNA and protein. Epigenetic changes have been implicated in both periodontitis and APOs (256-259), although they have been more well characterized with regards to PTB than LBW. Epigenetics may explain why periodontitis shows strong heritability, but genome-wide association studies have largely failed to find reliability reproducible genes responsible for periodontitis susceptibility. Most known epigenetic modifications that affect

periodontitis risk are in immune regulating genes (256). Traditional Mendelian gene variations in immune regulating genes also increase risk of periodontitis (44). The differences in susceptibility to *P. gingivalis*-mediated low fetal weight between C57BL/6 substrains may have been epigenetically "programmed" into the mice due to the environment at their respective vendor facilities.

Future Directions

Evaluate Tissue-Resident *P. gingivalis*-specific $CD4^+$ T Cells in Placenta

We were unable to evaluate the role of tissue-resident $CD4^+$ T cells in *P. gingivalis*-mediated fetal weight outcomes due to the low numbers of these cells detected in placental tissue. Single-cell RNAseq analysis of individual *P. gingivalis*-specific T cells sorted from placental tissue using the MHC class II tetramer may allow for a future correlation between frequency of tissue-resident *P. gingivalis*-specific $CD4^+$ T cells, including Treg cells, and low fetal weight in C57BL/6 mice. The major hurdle to this objective remains difficulty separating a small number of T cells from large volumes of placental tissue. Numerous separation gradients have been tried, yet placental tissue debris remains a major contaminant. Trypsin digestion of the placental tissue is often used to study other placental cell types, but appears to reduce the ability to stain for key T cell markers as compared to digestion with collagen D.

C57BL/6J and C57BL/6NCrl mice: Determining the Role of Microbiome in *P. gingivalis*-mediated Low Fetal Weight

Acting as a keystone pathogen, *P. gingivalis* is known to cause changes in nutrient availability in the gingiva. This altered nutrient environment shifts the normal symbiotic oral microbiota to become more dysbiotic and ultimately more pathogenic for the host. If *P. gingivalis* behaves similarly in the placenta, the key to why *P. gingivalis* induces low fetal weight in only some substrains of C57BL/6 mice may lie in the immune response to the pathobionts within the placental microbiota and not to *P. gingivalis* itself. Pathobionts in the normal microbiota are in sufficient low frequency not to induce pathology. The altered nutrient base initiated by *P. gingivalis* may be changing the prevalence or behavior of such pathobionts of the mouse oral or placental microbiome.

We thus hypothesize the presence of a microbial species in the normal mouse microbiota of C57BL/6J and UofM mice that is necessary in tandem with *P. gingivalis* to induce low fetal weight. Alternatively, there could be a species that is protective against *P. gingivalis*-mediated low fetal weight in C57BL/6NCrl mice.

This theory could be tested in two ways. First, C57BL/6J and UofM mice could be co-housed with C5BL/6NCrl mice prior to experimentation. If there is a bacterial species that makes C57BL/6J and UofM mice susceptible to low fetal weight, C57BL/6NCrl co-housed mice would experience reduced fetal weight. If there is a protective species in C57BL/6NCrl mice, the opposite would be

observed. If the microbiome plays no role and the differences in fetal weight are driven by substrain specific genetic differences, then co-housing would have no effect on fetal weight outcomes.

This theory could also be tested by treating C57BL/6J and U of M mice with different antibiotics prior to oral colonization with *P. gingivalis* to determine if depletion of specific populations of microorganisms could protect against *P. gingivalis*-mediated low fetal weight. The same antibiotics would be delivered to C57BL/6NCrl mice to see if depletion of a specific population induces *P. gingivalis*-mediated low fetal weight. This strategy was used to identify SFB as a major driver of Th17 SI-LP cell differentiation (260-265).

Microbiome sequencing assays to determine the difference between “fetal weight reduction permissive” and “fetal weight reduction prohibitive” microbiome populations could further pinpoint the relevant species in either experimental design. Alternatively, it must be considered that a change in the metabolic or enzymatic profile of the microbiome, and not an individual species, may be responsible for why only some substrains of C57BL/6 experience low fetal weight in response to *P. gingivalis*.

It is possible that $CD4^+$ T cells producing IFN-γ are responding not to *P. gingivalis* itself but to microorganisms that change in abundance because of *P. gingivalis*-driven changes in the nutrient foundation. Identification of co-species that are essential for *P. gingivalis*-mediated fetal weight reduction may identify a

novel population of $CD4^+$ T cells producing IFN-γ that correlate with low fetal weight in C57BL/6J and C57BL/6UofM dams.

This set of experiments may identify a species, or several species, or metabolic functions of a set of species that are necessary for *P. gingivalis*-mediated low fetal weight.

If the microbiome is determined not to be responsible for the difference between fetal weight outcomes in C57BL/6J and UofM mice as compared to C57BL/6NCrl mice, then the difference must be driven by either genetic or epigenetic changes. Most likely, both the microbiome and the genome are involved in *P. gingivalis*-mediated low fetal weight. This is demonstrated by the fact that *P. gingivalis* is detected in the placentas of mice both susceptible (C57BL/6J, UofM) and resistant (BALB/c) to low fetal weight. Understanding the proportional difference in the placental microbiome of fetal weight resistant or susceptible mice is an essential preliminary step before probing the genetic differences in *P. gingivalis*-mediated fetal weight outcomes occurring in different murine strains.

Determining the Role of the Innate Immunity in *P. gingivalis*-mediated Low Fetal Weight

Different SNPs can distinguish C57BL/6J from C57BL/6N mice. Whether these SNPs or other genetic variations influence *P. gingivalis*-mediated low fetal weight remains unknown. Genetically encoded differences in C57BL/6J,

C57BL/6N, and BALB/c mice may differentially regulate the innate immunity in response to *P. gingivalis*. For example, differential expression of certain TLRs is associated to APOs in BALB/c but not C57BL/6 mice after infection with *Ureaplasma parvum* (240). It is plausible that a difference in TLR expression also drives APOs in C57BL/6 but not BALB/c mice after oral and placental colonization with *P. gingivalis*. TLR and complement show strain-specific differences in function and interact with *P. gingivalis* virulence factors in a way that favors *P. gingivalis* growth and survival. Substrain-specific differences in TLR and complement between C57BL/6J and C57BL/6N mice have not been evaluated in the current literature.

Strains of *P. gingivalis* that lack the key virulence factors for interacting with TLR and complement have been developed (Kgp-deficient (KDP 129); Rgp-deficient (KDP 133); or Kgp- and Rgp-deficient (KDP 136)) (266-270). Oral colonization of C57BL/6J mice with *P. gingivalis* that lack the key virulence factors that interact with TLR or complement can dissect the contribution of *P. gingivalis* to low fetal weight.

TLR or complement mutant mice could also be used to elucidate the role of innate immunity in *P. gingivalis*-mediated low fetal weight. Alternatively, BALB/c and C57BL/6 mice could be crossed to create F1 offspring. It could then be determined whether F1 hybrids have a fetal weight phenotype in response to *P. gingivalis* that is more similar to the BALB/c or the C57BL/6 parent. Similarities and differences in complement and TLR gene expression and fetal

weight phenotype between the F1 and its two parents could then include or exclude their involvement in *P. gingivalis*-mediated fetal weight outcomes.

Conclusions

Removing or reducing the amount of *P. gingivalis* and other oral pathogens from the periodontal pocket alone through SRP therapy does not reduce the risk of APOs in women. The prevalence of APO in women with periodontitis points to a potential role for local inflammation of placenta due to colonization with *P. gingivalis* alone or with other oral pathobionts capable to persist in the placenta despite therapy around teeth.

After oral colonization, C57BL/6J and C57BL/6UofM mice experience low fetal weight, but C57BL/6NCrl do not. *P. gingivalis* seeding of the placenta is correlated to low fetal weight only in C57BL/6J and C57BL/6UofM mice and not in C57BL/6NCrl. BALB/c mice regardless of vendor did not experience low fetal weight despite presence of *P. gingivalis* in the placenta. The immune response that potentially ensues against placental *P. gingivalis* or against other pathobionts leading to low fetal weight is *not* related to $CD4^+$ T cells specific for *P. gingivalis* recirculating to the PaLN-CLN. Specifically, production of IL-17A, IFN-γ, or IL-4 by *P. gingivalis*-specific $CD4^+$ T cells in the PaLN-CLN cannot explain why low fetal weight occurred in C57BL/6J and C57BL/6UofM mice.

These findings suggest a role for both environmental and genetic factors in *P. gingivalis*-mediated low fetal weight. Future experiments should be

designed to understand which components of the microbiome lead to *P. gingivalis*-mediated low fetal weight in C57BL/6J and UofM but not C57BL/6NCrl mice. There may also be genetic differences between mice on the C57BL/6J (including UofM) background versus C57BL/6N or BALB/c mice that lead to *P. gingivalis*-driven low fetal weight, with or without a simultaneous contribution of the microbiome. Evaluation of genetic components of the innate or adaptive immune system that vary between strains of mice may answer the question of why some substrains of C57BL/6 but no substrains of BALB/c mice are susceptible to *P. gingivalis*-associated low fetal weight. Particularily, differences in complement and toll-like receptor function will be evaluated, as these innate systems are known to differ in C57BL/6 versus BALB/c mice, interact with *P. gingivalis* virulence factors and have an impact in APOs.

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